PHYTOPATHOLOGISCH LABORATORIUM

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BAARN

MEDEDELING No. 28

THE INFLUENCE OF ANTAGONISTIC FUNGI ON THIELAVIOPSIS BASICOLA (BERK. ET BR.) FERRARIS

BY

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REPRINTED FROM: ACTA BOTANICA NEERLANDICA 9: 59-118, 1960

COMMONWEALTH MYCOLOG

Acta Botanica Neerlandica **9** (1960) 59–118

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(*Received November 14th, 1959*)

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1. INTRODUCTION

Although *Thielaviopsis basicola* (Berk. et Br.) Ferraris can not be regarded as a severe parasite, the root-rot which it calls forth, may nevertheless cause considerable damage to certain host plants, in the field as well as in greenhouses. The fungus appears to be distributed in various kinds of soil, and almost all growers of root-rot susceptible plants become at one time or another confronted with it. Nevertheless, the presence of this root-parasite in the soil does not necessarily imply that susceptible plants will be severely attacked; in the same soil and with the same plant species the degree of injury may differ from year to year.

The variability in the pathogenicity of *Thielaviopsis basicola* has usually been ascribed to the influence exercised by physical factors either on the parasite itself or on the host plant. Conditions that are unfavourable to the development of the host, may give the fungus a better opportunity of attack, whereas conditions that are unfavourable to the fungus, may decrease its ability to penetrate into the tissues of a susceptible plant. A non-physical factor unfavourable to the development of *Thielaviopsis* in the soil might be the presence of micro-organisms that would be able to act as antagonists. It is rather striking that the influence of such organisms on *Thielaviopsis*, and indirectly on the disease caused by the latter, has so far received little or no attention. For this reason it looked attractive to study the influence which such micro-organisms, and especially some antagonistic fungi, may exercise on *Thielaviopsis*, in vitro as well as in the soil. In addition in two different soils the relation between the natural microflora and the population of *Thielaviopsis* was investigated during a period in which the soil was infested with the latter at regular intervals.

Out of a great many fungi that were isolated from different soils, a certain number proved to be antagonistic against *Thielaviopsis*, and among the latter some were selected for further study. Their antagonistic activity was measured by the aid of the effect which was exercised on the growth of *Thielaviopsis* by filtrates of the media in which they had been grown. The effect of external conditions, such as the nature of the medium in which the antagonists were cultivated, was also investigated. The effect of the antagonists on the pathogenicity of *Thielaviopsis* in the soil could be determined by estimating the severity of the disease symptoms shown by a sui-

table host. For this purpose *Nicotiana glutinosa* L. was chosen, because it is very susceptible to infection by this root parasite.

As far as is known to the author, no experiments have hitherto been carried out in this context with *Nicotiana glutinosa*. This plant was mentioned for the first time as a host of *Thielaviopsis* by JOHNSON (1916). When grown in soils that were heavily infested with tobacco black root-rot, its susceptibility appeared to be high. That the infection with *Thielaviopsis* has received no further attention, probably finds its explanation in the fact that this host was not considered to be of much, if any, economic importance. However, during the past few years it has become an important test object in the study of tobacco-mosaic virus.

As *Nicotiana glutinosa* appeared to be very sensitive to external conditions such as light intensity and temperature, the effect of a variation in these conditions on its development had to be known before an assessment of the pathogenicity of *Thielaviopsis* could be undertaken. This was the more necessary as adverse conditions of growth may cause symptoms which are very similar to those caused by the infection. It might also be that plants which did not show any symptoms of decline caused by physical factors, had decreased in their sensitivity to *Thielaviopsis*. In order to test this possibility the plants that were exposed to different combinations of external conditions, were grown partly in a soil that was infested with *Thielaviopsis*, and partly in a soil that was not infested. For further experiments that combination of conditions was chosen which had proved to be as favourable as possible for a good growth of the plants and which still allowed an accurate assessment of the severity of the disease symptoms caused by *Thielaviopsis basicola*.

2. SOME REMARKS ON THE HISTORY OF THE SUBJECT

2. 1. ON THIELAVIOPSIS BASICOLA AS CAUSE OF THE "BLACK ROOT-ROT" AND ON VARIOUS FACTORS AFFECTING THIS DISEASE

A cursory survey of the literature dealing with *Thielaviopsis basicola* (Berk. et Br.) Ferraris and with the "black root-rot" which is caused by this fungus, will suffice to convince us that this parasite, which was described for the first time in 1850 by BERKELEY and BROOME under the name *Torula basicola*, has already drawn considerable attention.

In the earlier publications there is often some confusion with regard to its identity, because this fungus was regarded for some time as the conidial stage of an ascomycete described in 1876 by ZOPF as *Thielavia basicola*. On account of this mistaken identification the fungus of BERKELEY and BROOME received in 1912 from FERRARIS the name *Thielaviopsis basicola*. However, in 1925 MCCORMICK could prove that the fungus of which the perithecial stage had been described by Zopf as *Thielavia basicola*, is a distinct species, and that the association with the imperfect fungus *Thielaviopsis basicola* is but accidental. Further evidence in support of this view was provided by LUCAS (1948) and by HÄRRI (1959).

Thielaviopsis basicola is able to infect a large range of plants. Already in 1916 more than a hundred species belonging to 18 families were, according to Johnson, known as hosts, and since then their number has still further increased. Garrett (1956) regards *Thielaviopsis basicola* as a primitive, unspecialized parasite, and he adds that although strains of different virulence occur, they all seem to agree in the wide range of their hosts. With regard to the damage caused by this fungus it may be remarked that it is really destructive only to young plants and to plants growing under somewhat adverse conditions.

Thielaviopsis basicola occurs in different soils. Yarwood (1946) collected in 12 Californian localities 17 soil samples, and succeeded in isolating *Thielaviopsis basicola* from 12 of them; the latter were obtained from 7 of the 12 localities. He further found that the fungus was present in soils where crops were grown which showed no symptoms of the disease. Stover (1950b) reported that *Thielaviopsis* was prevalent in several fields that were situated over 100 miles from the old tobacco areas, and on which as yet no more than three tobacco crops had been grown. For this reason he assumes that this fungus must be able to live in soils on which no susceptible plants occur. Under such circumstances it must be able to persist partly as a weak parasite and partly as a saprophyte until the introduction of a highly susceptible host offers it the opportunity to develop its full parasitic vigour. The possibility of a survival in susceptible weeds is not considered by him. An inquiry instituted in the Netherlands by Mooi–Bok (1952) revealed that the fungus has been found here nearly everywhere where *Lathyrus odoratus* is grown.

Special studies have revealed that the effect exercised by *Thielaviopsis basicola* on susceptible plants is variable, and that this variability depends on external conditions. The influence of the latter on the black root-rot of *Nicotiana tabacum* has been thoroughly investigated, but little or no research has been carried out so far on the black root-rot of *Nicotiana glutinosa*, the plant that was used as host in most of our own investigations. However, a brief survey of the studies that have been carried out with *Nicotiana tabacum* as host, will be of interest.

From the work of Johnson and Hartman (1919) on the influence of the soil factors it is clear that in tobacco the severity of the black root-rot is determined in the first place by the temperature of the soil. Relatively low temperatures favour the development of the disease, but higher temperatures are less suitable for it; above 26° C the infection rapidly declines, and at about 30° C hardly any trace of the infection is left. Similar results were obtained by Valleau, Kenney and Kinney (1925). They report that at soil temperatures between 21° C and 23° C black root-rot is a serious menace to tobacco, but that at an average soil temperature of 25.5° C the crop suffered hardly at all. The findings of Doran (1929) and of Jewett (1938) point in the same direction. The latter found that at temperatures between 18° C and 20° C the number of tobacco varieties that showed symptoms of the disease, was larger, and that the infection was much

more severe than it appeared to be at temperatures between 28° C and 30° C. According to Stover (1950b), the range of temperature which is most suitable for the estimation of the pathogenicity of *Thielaviopsis basicola*, would be found between 18° C and 25° C.

According to Conant (1927) the differences in resistance to the attacks of *Thielaviopsis basicola* that are shown by tobacco at various temperatures, would be due to differences in its capacity to produce a layer of cork in the tissue underlying the lesion. Jewett (1938), however, could not confirm these findings of Conant.

Infection experiments (Gilbert, 1909; Johnson and Hartman, 1919; Lucas, 1955, and others) showed that the most favourable temperature range for the infection is 17° C to 23° C, and that it does not correspond therefore with the optimum temperature found for the growth of the fungus in pure cultures nor with the optimum temperature for the growth of the host, in both cases 25°–30° C.

Investigators generally agree that a pH exceeding a value of approximately 5.6 is favourable to the development of black root-rot in tobacco. Briggs (1908) concluded from his experiments that substances which render the soil more alkaline, cause an increase of the black root-rot, whereas addition of substances with an acidifying effect cause a decrease of the disease. The results obtained by Johnson and Hartman (1919) with pot experiments point in the same direction. In the field experiments carried out by Anderson, Osmun and Doran (1926) it was found that black root-rot caused little or no damage to the tobacco crop when the pH of the soil remained below 5.6, whereas the damage became severe when the pH of the soil exceeded 5.9. The critical range, therefore, lies between pH 5.6 and pH 5.9. The position of this critical range is not fully constant, but may shift somewhat under the influence of the temperature and of other factors. They studied the influence of the pH also in pure cultures of *Thielaviopsis basicola*; the results obtained with the latter proved to be in agreement with those arrived at in the field experiments. The results obtained by these investigators were confirmed by Anderson and Morgan (1926) and by Morgan and Anderson (1927).

Doran (1929) found that there was little or no black root-rot so long as the pH of the soil remained below 5.6, no matter what the temperature was. However, the point at which the pH began to allow the development of the disease, appeared to be markedly influenced by the temperature. At 15° C the disease made its appearance at a pH of 5.7, at 18° C at a pH of 5.7–5.8, at 21° C–24° C at a pH of 5.8, at 27° C at a pH of 5.8–5.9. At a temperature of 30° C there was hardly any injury at all, even when the pH reached values ranging from 6.0 to 6.9. The critical pH-value, therefore, appeared to increase with the temperature. In a later report (1931) he confirmed the findings of Briggs by showing that the infection by *Thielaviopsis* decreased when the pH of a limed soil was subsequently lowered by acidification.

Next to the temperature and the pH, the moisture content of the

soil is of importance. GILBERT (1909) concluded from his experiments that excessive watering is favourable to the development of black root-rot in tobacco. JOHNSON and HARTMAN (1919) confirmed his findings, and gave his conclusion a somewhat more precise form by stating that the soil humidity is of importance only when the soil is very wet or when it is saturated with water; under such circumstances infection by *Thielaviopsis basicola* is more severe. With regard to the humidity of the air no experiments have as yet been performed.

JOHNSON and HARTMAN (1919) and ANDERSON, OSMUN and DORAN (1926) studied the relation between the degree of black root-rot of the tobacco crop and the amount of *Thielaviopsis* inoculum that is present in the soil. They agree with each other that the degree of damage is directly proportional to the number of places at which the roots become infected. In order that the disease may assume a serious form, it is therefore necessary that the soil contains a high amount of inoculum. More precise data were supplied by LEVYKH (1938). He studied the problem by means of pot experiments, and found that tobacco seedlings proved to be but very slightly infected and that the number of them that could be transplanted, was but slightly reduced when the soil was infested with less than 100 chlamydospores per cc soil; that adult tobacco plants were slightly infected when the soil was infested with 100–1000 chlamydospores per cc; that a severe infection was obtained when the number of chlamydospores was raised to 3.312 per cc, and that with 5.780 chlamydospores per cc the highest degree of infection was reached. STOVER (1950b) too found that the effect of an infestation is markedly influenced by the amount of inoculum. If the latter is either very small or very high, it is impossible to determine the pathogenicity of the strain that is used for the infestation, from the effect it produces; if the amount exceeds a certain level, the effect remains the same, and if the amount remains below a certain level, there is no effect at all.

The authors whose publications were reviewed above, have shown that the pathogenicity of *Thielaviopsis basicola* may be influenced by several factors, soil temperature, pH of the soil, quantity of inoculum that is available, character and condition of the host, etc.

However, there is one factor that has been left out of consideration by previous investigators, and of whose influence on *Thielaviopsis basicola* as yet little is known. This is the presence of antagonistic micro-organisms. As a background for the investigations to be carried out on the influence exercised by such organisms on *Thielaviopsis basicola*, it seems appropriate to consider some general aspects of microbiological antagonism.

2.2. ANTAGONISM BETWEEN MICRO-ORGANISMS AND THE SIGNIFICANCE OF THIS PHENOMENON TO PHYTOPATHOLOGY AND SOIL MICROBIOLOGY

The existence of an antagonism between various micro-organisms was known already towards the end of the preceding century, but it was especially during the last decades that it became an object of

more general interest to microbiologists as well as to phytopathologists and medical men. Several handbooks dealing with this topic have already been published, e.g. WAKSMAN (1945), HERRELL (1945), FLOREY *et al.* (1949), VOGEL (1951). Several times too the subject has been reviewed in journals, e.g. by WAKSMAN (1937), WEINDLING (1946), BRIAN (1951), WOOD and TVEIT (1955). The number of special papers has increased to such an extent that in this short survey but a small part of them can be mentioned.

2.2.1. *Antagonistic micro-organisms and their products*

POTTER (1908) found that *Pseudomonas destructans* Potter (= *Erwinia carotovora* (Jones) Holland), the cause of the "turnip rot", produced a substance by which this organism itself may be killed. This substance proved to be heat-resistent and more or less specific for the organism by which it is produced. It appeared, moreover, that the "turnip rot" could be combated by treating the turnips with this toxin. Potter therefore was one of the first to make use of microbial antagonism in the control of a plant disease. In a similar way it appeared possible to control *Penicillium italicum* Wehmer, which causes a disease in oranges, by means of a substance produced by this fungus in its own metabolism. Potter supposes that the faculty to produce such substances may be more generally distributed, and that it may provide us with a means to control some of the plant diseases.

Another important contribution to our knowledge of microbial antagonism was made by PORTER (1924). The latter found that the growth of *Helminthosporium* may be inhibited by various kinds of bacteria, and he could show that one of these bacteria produced a diffusible substance which inhibited the growth of *Helminthosporium* already at a distance of 2 cm from the place where it was produced. A distinct protection of wheat and flax seedlings against infection by *Helminthosporium* and *Fusarium* respectively was obtained by the use of such antagonistic bacteria.

BAMBERG (1930) reported that he had succeeded in isolating from maize plants a bacterium which inhibited the infection of these plants by *Ustilago zeae* (Beckm.) Unger and which destroyed the galls caused by this fungus when the latter had already been developed.

That micro-organisms which are normally present in the soil, may exercise an inhibiting effect on a soil-borne disease, was recognized by HARTLEY (1921). This author carried out infection experiments with *Pythium debaryanum* Hesse, and found that the conifer seedlings used for these experiments showed much more "damping off" when the soil in which they were grown, was previously sterilized, than when it was left unsterilized and that the disease could, to a large extent, be kept in check by an infestation of the soil with saprophytes like *Rhizopus nigricans* Ehrenb.

HENRY (1931) made a similar observation with regard to the pathogenicity of *Helminthosporium sativum* Pk., the cause of "foot-rot" in wheat. This parasite may be kept in check by means of various soil microbes; the more saprophytic organisms were added to the

sterilized soil, the greater the antagonistic effect on *Helminthosporium* appeared to be.

TIMS (1932) could show that various actinomycetes which were isolated by him from sugar-cane soils, exercise a strong antagonistic effect on a *Pythium* that grows as a parasite on sugar-cane. In infection experiments that were carried out in sterilized soil with sugar-cane as well as with wheat, the root-rot of these plants could be reduced by inoculating the soil with a culture of one of the more active antagonists.

In test-tube experiments with cabbage seedlings grown from sterilized seeds, JAARSVELD (1942) found that various soil fungi are able to reduce the pathogenicity of *Rhizoctonia solani* Kühn. All soil fungi that were tested in these experiments, proved to exercise an antagonistic action, and when more than one of these fungi were added, the effect proved to be increased. Experiments with potted plants led to more or less similar results.

A noteworthy contribution to our knowledge was brought by the paper of VAN LUYK (1938). He found that various fungi may exercise an antagonistic action on species belonging to the genus *Pythium*; and the effect which culture filtrates of *Pullularia pullulans* (de Bary) Berk. and of *Penicillium expansum* (Link) Thom exercise on the growth of *Pythium debaryanum*, was estimated quantitatively in pure cultures of the latter. Remarkably effective were the filtrates obtained from *Penicillium expansum;* even in a dilution of 1 to 1280 they inhibited the growth of the parasite. VAN LUYK's greenhouse experiments with grass and lucerne showed that the infection of these plants by *Pythium debaryanum* was strongly reduced by the activity of the antagonists. He also studied the way in which the antagonistic activity of the filtrate of *Penicillium expansum* is influenced by the carbon source present in the culture medium, and investigated some of the properties shown by the metabolic products of this antagonist. The antibiotic "expansin" that is involved here, was isolated by OOSTERHUIS in 1946.

SLAGG and FELLOWS (1947) tested the effect of 143 different kinds of soil fungi on *Ophiobolus graminis* Sacc., and found that about one quarter of them exercised an antagonistic action. In pure cultures these antagonists formed "by-products" which proved to inhibit the growth of *Ophiobolus graminis*, and in artificially as well as in naturally infested soil several of them were found to decrease the damage caused by this parasite to wheat. Similar instances of antagonism between soil fungi and soil-borne plant parasites have repeatedly been reported in subsequent years (COOPER and CHILTON, 1950; LUKE, 1952a, 1952b; JOHNSON, 1952, 1954; etc.).

A large part of the investigations on the antagonism between micro-organisms have been carried out either in the laboratory by means of pure cultures or else in the greenhouse, where the host plants were grown in a sterilized and subsequently artificially infested soil. However, it is not a priori certain that antagonism will play a similar rôle in natural soils with their complex microbiological popu-

lation. GARRETT (1956), who in his handbook "Biology of root-infecting fungi" has summarized his own observations as well as those of other investigators, points out that the intricate composition of the microbial population in natural soils is to be regarded as one of the factors which tends to obscure the effect of the antagonism which is observed in pure cultures and in sterilized soil after an artificial infestation.

Several investigators have noted that the soil fungi belonging to the genus *Trichoderma* are of importance as producers of antibiotic substances. In 1932 WEINDLING described the way in which the hyphae of *Rhizoctonia solani* and of other fungi were parasitized by *Trichoderma lignorum* (Tode) Harz, and BISBY, JAMES and TIMONIN (1933) and BROWN (1933) observed the parasitism of this fungus on *Fusarium culmorum*, on *Helminthosporium sativum* and on *Phymatotrichum omnivorum*, and remarked that it might aid in controlling the phytopathogenic fungi living in the soil. Some other observers too have noted the antagonistic action of *Trichoderma*, e.g. BUTLER (1935), ALLEN and HAENSELER (1935) and DAINES (1937).

WEINDLING had noted already in 1932 that in a culture medium *Rhizoctonia solani* may be destroyed by a substance or by substances secreted by *Trichoderma lignorum*. In 1934 he published a paper dealing with the "lethal principle" produced by this fungus; some of the properties of the latter were described, and the author added that there is evidence that this lethal principle is a single chemical substance. Two years later WEINDLING and EMERSON (1936) announced the isolation of an antibiotic from the culture filtrate of *Trichoderma lignorum*, and described the chemical properties of this substance, which in later papers of WEINDLING (1937, 1941) is called "gliotoxin". The production of this antibiotic by *Trichoderma* was studied also by BRIAN (1944), BRIAN and HEMMING (1945) and others.

Subsequently BRIAN and McGOWAN (1945) and BRIAN, CURTIS, HEMMING and McGOWAN (1946) succeeded in isolating another antibiotic from strains of *Trichoderma viride* Pers. ex Fr. This substance, for which they proposed the name "viridin", is a stronger antibiotic than gliotoxin, but it is less stable. Afterwards BRIAN (1951) found that most strains of *Trichoderma viride* produce a mixture of gliotoxin and viridin.

2.2.2. *Production and rôle of antibiotics in the soil*

Earlier studies have led to the discovery of a number of antibiotics of which in 1951 BRIAN has given a summary. It appears from the latter that 2191 species of fungi belonging to 245 genera had already been tested, and that 785 species belonging to 120 genera had been found to produce active substances. Moreover, some 96 antibiotics had already been recognized, and 57 of the latter had been obtained in a pure form and are well characterized. The remainder were known only in crude extracts. In an earlier work BRIAN (1949) had suggested that "the capacity to produce antibiotics is particularly characteristic of micro-organisms whose natural habitat is in the soil,

and that this capacity may be in some cases a factor concerned in the maintenance or change of the microbiological balance in the soil, and thus indirectly concerned with soil fertility."

An extensive study on the production and rôle of antibiotics in the soil was started in 1951 by SIMINOFF and GOTTLIEB. One of their aims was to find out whether antibiotic substances are produced in the soil, and if so, whether they exert any influence on its microbiological population. This work was continued by GOTTLIEB and SIMINOFF (1952), MARTIN and GOTTLIEB (1952), GOTTLIEB, SIMINOFF and MARTIN (1952) and MARTIN and GOTTLIEB (1955). They studied the production in the soil of 11 antibiotics formed by well-known antagonists, and investigated the part played by these substances and their ultimate fate. They arrived at the conclusion that some of them are soon inactivated, for instance by adsorption to the soil particles. Some other ones, however, appeared to play a more active rôle in the life of the microbiological community.

JEFFERYS (1952) has studied the stability of 10 antibiotics in different soils. In contrast with the investigators quoted above, he added solutions of antibiotics to the soil. He found that the antibiotics differed from each other in their stability, and that the latter varied from soil to soil. Nevertheless, in some of the soils they all exhibited a fair degree of stability. Their inactivation rests in his opinion on four causes, viz. (1) an unfavourable pH of the soil, (2) adverse microbial activity, (3) their adsorption to soil particles, and (4) an as yet unknown factor, possibly of a chemical nature.

NISSEN (1954) studied the effect exercised by high concentrations of antibiotics on the complex micropopulation found in the soil. In order to obtain an idea of the changes in microbiological activity in the soil he estimated the production of carbon dioxide in the latter, as he regarded the production of this substance as a measure of the microbiological activity. He experimented with five antibiotics, and found that they differed in the rate at which they were inactivated in the soil.

The results obtained by JEFFERYS, BRIAN, HEMMING and LOWE (1953) seem to lend support to the view that the production of antibiotics is of ecological importance to the fungus flora of the soil. They found that 45 % of the wide-spread and locally abundant fungi of acid heath soils were able to produce these substances, whereas but 15 % of the rarer fungi were able to do so.

The findings of DOBBS and HINSON (1953) indicate that in natural soils a "fungistasis" is of common occurrence. This view is shared by JEFFERYS and HEMMING (1953), who are of opinion that this may find its explanation to some extent in the activity of the antibiotics. They add that their observations suggest the presence of discontinuous "islands" of inhibition rather than that of a wide-spread "sea" of inhibition. The presence of inhibitory material in the soil was confirmed by HESSAYON (1953), CHINN (1953), STOVER (1955, 1958) and others.

The way in which the inhibitory substances affect the spores and

hyphae of the fungi, has been studied too. Park (1955, 1956) observed a lysis of fungus spores in contact with soil particles. Jackson, who reported in 1958 on the presence of a fungistatic factor in Nigerian soils, tested the fungistatic effect of the soil on 19 species of fungi, and found that in 11 of them the germination of the spores was distinctly inhibited. Lockwood (1959) states that natural loam causes in various fungi a lysis of the mycelium, and inhibits the germination of the conidia. He ascribes this to the presence of lytic and toxic substances produced by species of *Streptomyces*, and he succeeded in demonstrating the presence of such substances in the soil.

The findings of the investigators quoted above indicate that fungitoxic substances are present in the soil, and that the latter doubtless play an important part in the maintenance of the microbiological balance in that habitat.

2.2.3. *Antagonism against Thielaviopsis basicola*

The results obtained in the study of microbial antagonism suggest that the differences in pathogenicity shown by *Thielaviopsis basicola* may to some extent be due to the activity of the antagonistic microorganisms by which it may be accompanied. However, so far but little was known of the influence which such organisms may exercise on this parasite. Tiddens (1933) found that *Primula obconica* is more severely infected by this fungus when it is grown in sterilized soil than when it roots in an unsterilized one. Stover (1950a) reports that in cultures of this root parasite the growth of the latter was sometimes inhibited by the presence of an unidentified bacterium. Mooi–Bok (1952) observed that in cultures of *Lathyrus odoratus* the infection by *Thielaviopsis basicola* decreased when *Fusarium oxysporum* Schl. was added to the soil. She also carried out experiments on the direct influence of "antagonists" on *Thielaviopsis basicola* in order to find out whether they might be used for a biological control of the latter, but the results were on the whole negative.

3. ON THE ISOLATION OF THIELAVIOPSIS BASICOLA AND ON THE SELECTION OF SUITABLE ANTAGONISTS

3.1. Isolation of thielaviopsis basicola

Diseased *Primula obconica* plants, infected by *Thielaviopsis basicola*, were examined macroscopically as well as microscopically. The disease symptoms caused by this parasite are readily recognizable; one is a yellow discoloration which comprises the whole leaf surface with the exception of a narrow zone along the principal veins; another symptom is to be found in the brown to black discoloration of the rotting roots. In the infected parts of the root system chlamydospores of the parasite proved to be present in large amounts. In order to isolate the pathogen, fragments of the diseased roots were superficially sterilized and laid out on cherry agar. In many instances not only *Thielaviopsis basicola*, but also species of *Pythium*, *Fusarium*

and *Cylindrocarpon* were found to grow out from the root fragments. However, it was not difficult to obtain *Thielaviopsis basicola* in pure culture.

When *Nicotiana glutinosa* was infected with *Thielaviopsis basicola*, the diseased roots soon turned brown, and in that case they showed numerous lesions ranging in colour from brown to black. Contrary to what happens in *Primula obconica*, not only the young roots appear to be affected but also the older ones which are often entirely covered by large black, slightly swollen patches. The taproot may show the symptoms of the disease right up to the base of the stem. The young roots are probably soon killed by the parasite, and for this reason it is more difficult to isolate *Thielaviopsis basicola* from *Nicotiana glutinosa* than from *Primula obconica*. The older roots nevertheless contain not only the dark-coloured mycelium of the parasite but also large numbers of chlamydospores. A successful method for isolating *Thielaviopsis basicola* consisted in rinsing the diseased roots in running tap-water for at least 15 hours, dipping them for a few seconds in 96 % alcohol, and subsequently washing them in sterilized water; then the roots were dried on sterilized filter-paper, divided in small parts, and laid out on cherry agar. When the cultures were 2–3 days old, spore suspensions were made of them, and with the latter pure cultures of the parasite could be obtained. In most instances not only *Thielaviopsis* but *Fusarium* too developed from the roots that had been laid out, and this was a great drawback, for it proved particularly difficult to separate *Thielaviopsis basicola* from this fungus. It does not seem impossible that *Fusarium* too may impart a disease to *Nicotiana glutinosa*.

A modification of the carrot-disc method introduced by Yarwood (1946) has also been used for the isolation of *Thielaviopsis basicola* from diseased roots of *Nicotiana glutinosa* (Stover, 1950a). The roots were thoroughly washed, macerated and spread out on the surface of fresh carrot discs. Within a few days greyish-green to almost black colonies of *Thielaviopsis basicola* appeared on the surface of the latter; the large masses of endoconidia that were formed by these colonies, could easily be transferred to Petri dishes or test tubes containing a suitable agar nutrient. This method proved to yield much better results than the method of superficial sterilization described above.

The method of Yarwood (1946) has also been used for isolating *Thielaviopsis basicola* from soil. Fresh, approximately 0.5 cm thick carrot discs were placed in Petri dishes, and covered with a 0.5 cm thick layer of soil taken from pots in which diseased tobacco plants had been grown. Then a slight pressure was exercised on the soil, and an amount of water was added sufficient to moisten the soil, but leaving no surplus in the Petri dish. After the Petri dishes had been kept for about three days at room temperature, the carrot discs were washed in order to remove the soil, and placed in sterile moist chambers that were kept in an incubator at approximately 23° C. After about 24 hours in the moist chambers several grey colonies of *Thielaviopsis basicola* were already distinguishable on the

surfaces of the carrot discs Microscopic examination revealed that at this stage an extensive mycelium was present with a large number of conidiophores and of endoconidia, but as yet without chlamydospores. Pure cultures therefore could already be obtained. After a sojourn of 3 to 4 days in the incubator the carrot discs proved to be entirely overgrown with the greyish-green to nearly black fungus, and although conidiophores and endoconidia still prevailed, chlamydospores too were now present in a considerable amount.

Soil samples taken from various gardens at Baarn have been tested in this way, and it appeared that *Thielaviopsis basicola* is rather common in this place. The method proved to be particularly suitable because it requires but little time, and so it could, for instance, easily be found out whether *Thielaviopsis basicola* is present in a sufficient amount to cause black root-rot in tobacco plants.

3.2. Isolation of antagonistic fungi

The name antagonist will here be used for those fungi which exercise unmistakably an inhibiting influence on the development of *Thielaviopsis basicola*, or which are able to cause lysis of the mycelium.

In order to obtain antagonists, the fungus flora of clay and sand soils as well as of other likely substrates, e.g. leaf-mould, was examined. At a later stage also "diseased" and "healthy" *Primula* soils, some of which had been periodically infested with *Thielaviopsis basicola*, were tested as to the presence of antagonists. Two different methods were applied for obtaining the latter.

In the first place suspensions were prepared of 10 soils, and for the analysis of the fungus flora of each soil 10 Petri dishes were set apart. The suspensions were mixed with molten cherry agar at 40° C and the mixture poured out in the Petri dishes. Immediately after the plating-out, the mixture was inoculated at 5 different spots with *Thielaviopsis basicola*. The suspension should be diluted so far that in each Petri dish 10–30 fungus colonies will develop. The pH of the medium should lie between 4 and 5.

When the second method was followed, Petri dishes with cherry agar, were used, in the center of which *Thielaviopsis basicola* was inoculated. After being incubated for 5 days at 24° C, each colony of *Thielaviopsis* was surrounded at 5 places with soil particles.

The second method is certainly not an ideal one, but it nevertheless yielded good results. A distinct advantage is that it saves time and material, which is especially important when a large number of soils have to be tested. It may be made even more effective by inoculating each agar plate with more than one colony. The principal disadvantage of this method is that the development of some of the fungi that are present in the soil particles, may be made impossible by that of the more vigorously growing ones, so that they find no opportunity to display their antagonistic action.

By means of these two methods altogether 38 fungi were obtained which on cherry agar exercised an inhibiting effect on the growth of *Thielaviopsis basicola*. Of these 38 fungi pure cultures were made.

3.3. Selection of the most active antagonists

Among the fungi that had been isolated by means of the two methods described in the preceding section, those whose culture filtrates displayed the strongest antagonistic activity with regard to *Thielaviopsis basicola*, were selected for further study.

In order to obtain the culture filtrates, the fungi were cultivated in cherry juice (50 cc per Erlenmeyer flask with a capacity of 300 cc). They were incubated for 20 days at a temperature of 24° C, after which the culture liquid was passed through filter-paper and then through a sterilized Seitz filter.

The culture filtrates that had been obtained in this way, were diluted by means of cherry juice to one half, one quarter or one eighth of their original strength, and these dilutions were divided in amounts of 10 cc over test tubes; in the latter they were inoculated with *Thielaviopsis basicola*; test tubes filled with 10 cc cherry juice were also inoculated, and served as control. The test tubes were kept in an incubator at 24° C.

In the test tubes which contained cherry juice without any addition of culture filtrate, the development of *Thielaviopsis basicola* was good; the fungus covered the culture liquid with a thick layer of mycelium; after about 8 days growth came to a stop. In the other test tubes the development of the fungus was sometimes equal to that in the control tubes, but in other instances it proved to be inhibited to a more or less considerable degree. About 20 % of the fungi that were tested in this way, proved to be markedly antagonistic. This applies, of course, only to fungi that are grown on cherry juice; it is not impossible that other values will be found when the experiments are carried out with filtrates obtained from other culture media.

In subsequent experiments use was made only of those fungi whose culture filtrates had proved to be most strongly inhibiting. These fungi are:

Aspergillus fumigatus Fres., strain 1
Aspergillus fumigatus Fres., strain 2
Penicillium spiculisporum Lehman
Penicillium spinulosum (Link) Thom
Penicillium expansum (Link) Thom
Penicillium roqueforti Thom
Gliocladium roseum Bain.
A sterile mycelium

In order to obtain more active strains of these fungi, of each of them 5 mono-spore cultures were made, and the effect of their culture filtrates on *Thielaviopsis basicola* was compared in experiments performed under the same circumstances. Here too the filtrates were obtained from cultures in cherry juice in the way described above. It appeared that the filtrates of some of these mono-spore cultures exercised a considerably stronger inhibiting effect than others did that were derived from the same original culture. The most active strains were kept in culture in order to serve in subsequent experiments.

4. ON THE ANTIBIOTIC ACTIVITY OF THE SELECTED ANTAGONISTS

The experiments described in this chapter were carried out in order to determine the influence exercised by various external factors on the production of substances which inhibit the development of *Thielaviopsis basicola.* In addition, the production of these substances by various strains of *Penicillium roqueforti* was compared, and also the effect of the culture filtrate of this fungus on different strains of *Thielaviopsis basicola.*

4.1. SURVEY OF THE LITERATURE DEALING WITH THE ANTIBIOTICS PRODUCED BY THE ABOVE MENTIONED ANTAGONISTS

That *Aspergillus fumigatus* may exercise an antagonistic influence, was demonstrated already in 1913 by VAUDREMER (quoted from WAKSMAN, 1945). Since then several antibiotics have been isolated from the culture filtrates of this fungus, viz. fumigatin (OXFORD and RAISTRICK, 1942; and others), spinulosin (OXFORD and RAISTRICK, 1942; and others), fumigacin, which proved to be identical with helvellic acid (WAKSMAN, HORNING and SPENCER, 1943; MENZEL, WINTERSTEINER and HOOGERHEIDE, 1944; WAKSMAN and GEIGER, 1944) and gliotoxin (GLISTER and WILLIAMS, 1944; MENZEL, WINTERSTEINER and HOOGERHEIDE, 1944).

Of *Penicillium spinulosum* it is known that it produces spinulosin, a substance isolated and studied by BIRKINSHAW and RAISTRICK (1931). Nothing has so far been reported on an antagonistic action of this *Penicillium* on other fungi.

Penicillium expansum has already repeatedly been mentioned as an antagonist of phytopathogenic fungi. VAN LUYK (1938) studied its antagonism with regard to *Pythium debaryanum* in vitro as well as in vivo, and discovered that it produces a particularly active culture filtrate. JAARSVELD (1942) used it as an antagonist for the control of *Rhizoctonia solani.* The antibiotic was isolated from the culture filtrate in 1946 by OOSTERHUIS, who called it expansin.

Little is known of the antagonistic activity of *Gliocladium roseum* itself, but another species of the genus *Gliocladium,* viz. *Gliocladium fimbriatum* Gilm. et Abbott, is known to produce gliotoxin (WEINDLING, 1937, 1941) and to exercise an antagonistic influence on other fungi.

Of an antagonistic activity of *Penicillium roqueforti* and of *Penicillium spiculisporum* nothing was known so far. In the tables published by FLOREY *et al.* (1949) these two fungi are mentioned among those that have been tested with regard to their antibiotic activity with negative result. By JEFFERYS, BRIAN, HEMMING and LOWE (1953), who studied the antibiotic activity of 65 fungus species isolated from acid heath soils, *Penicillium roqueforti* was tested in two different ways, but it was found to be inactive with regard to fungi as well as with regard to bacteria.

In view of the fact that so far nothing was known of an antibiotic activity of *Penicillium roqueforti* and of *Penicillium spiculisporum,* special attention was paid to these two fungi.

4.2. Production of antibiotics in organic media

In the experiments described above the antagonists were cultivated in a cherry-juice medium. However, as the composition of the medium in which they are cultivated, may influence the antagonistic activity of the culture filtrate, another series of experiments was carried out in which the same antagonists were cultivated in another medium, viz. in a potato extract.

In these experiments young and vigorously growing mycelium of each of the antagonists was transferred to a series of 7 Erlenmeyer flasks with a capacity of 300 cc. Each of the latter contained 50 cc of the sterilized cherry or potato extract. It was hoped that in this way of each antagonist at least 200 cc culture filtrate would become available. The culture flasks were kept in an incubator at 24° C for a period of 16 days; at the end of this period the culture liquid was filtered, and the filtrate diluted respectively to one half, one quarter, one eighth, one sixteenth and one thirty-second; part of it was used without diluting it.

Of each of the undiluted and of the variously diluted fractions of the filtrate, 5 samples of 20 cc were poured in Erlenmeyer flasks with a capacity of 100 cc. A control series consisted of 15 similar flasks filled with 20 cc of the original culture medium. For the inoculation were used round discs 3 mm in diameter taken from 10 days old cultures of *Thielaviopsis basicola*; the latter had been isolated from *Primula obconica*, and was cultivated on cherry agar. After the inoculation, the flasks were placed in an incubator with a temperature of 24° C, in which they were kept for a period of 14 days.

As a measure for the antagonistic activity of the filtrate it was decided to use the dry-weight of the mycelium. This was determined a short time before the controls had reached their maximum development, as with a longer period of growth the differences between the uninhibited and the variously inhibited mycelia would have been obscured. First of all, therefore, some experiments were performed in order to determine the length of the period which an uninhibited *Thielaviopsis* culture requires to reach its maximum development.

The contents of the flasks were filtered and the residuum washed and sucked dry; then it was dried for an hour at 70° C, and subsequently for 3 hours at 103° C; after cooling, the filters with the mycelium were weighed, and from the gross weight the previously determined weight of the filter subtracted. This does not yet give the dry-weight of the amount of mycelium produced during the period of growth; it still includes the dry-weight of the material that was used for the inoculation, and that of the particles of dust and of other insoluble material that may have been present in the filtrate and in the culture medium. In order to determine the weight of these contaminations the contents of a number of flasks in which no growth had taken place, was filtered, and the residuum treated in the same way as that obtained from the other flasks. By averaging the values found in this way, the dry-weight of the inoculation material and of insoluble particles derived from other sources was found, and so

the nett weight of the mycelium produced during the period of cultivation could be determined.

The results of experiments in which the antagonists had been cultivated in cherry juice, are given in Table 1.

TABLE 1

Dry-weight in mg of the mycelium of *Thielaviopsis basicola* developed in undiluted and diluted filtrates of the cherry-juice medium in which one of the listed antagonists had been cultivated

Antagonists	Degree of dilution of the filtrate					
	1	1/2	1/4	1/8	1/16	1/32
A. fumigatus I	0	0	0	0	0	50.0
A. fumigatus II	0	0	0	0	43.0	151.9
P. spiculisporum	0	0	0	22.7	112.5	185.2
P. spinulosum	0	0	0	87.5	161.5	223.7
P. expansum	0	0	0	0	0	0
G. roseum	0	142.4	239.5	245.0	266.8	272.0
P. roqueforti	0	0	26.6	148.5	179.5	219.5
"Sterile mycelium"	0	0	147.5	193.6	253.5	260.2

The average dry-weight of the control mycelia grown in cherry juice was 268.8 mg.

The effect of the culture filtrate of *Penicillium expansum* was tested also in higher dilutions. At a dilution of 1/128 still no growth was observed, at a dilution of 1/256 the dry-weight of the mycelium appeared to be 55.0 mg, and at a dilution of 1/512 it was 119.0 mg.

The culture filtrate of *Penicillium spiculisporum* caused in *Thielaviopsis* an entirely abnormal development; in most instances the mycelium formed a thin film at the bottom of the flask.

In the second set of experiments the antagonists were cultivated in a potato extract instead of in cherry juice. This medium is approximately neutral (pH 6.7–6.9) and rich in carbohydrate, whereas cherry juice is acid (pH ± 4.5) and poor in carbohydrate. The results of these experiments are given in Table 2.

TABLE 2

Dry-weight in mg of the mycelium of *Thielaviopsis basicola* developed in undiluted and diluted filtrates of the potato-extract medium in which one of the listed antagonists had been cultivated

Antagonists	Degree of dilution of the filtrate					
	1	1/2	1/4	1/8	1/16	1/32
A. fumigatus I	0	0	0	0	100.0	176.7
A. fumigatus II	0	0	0	94.0	172.1	184.5
P. spiculisporum	0	0	41.5	55.1	73.5	101.7
P. spinulosum	0	0	0	104.5	138.5	146.5
P. expansum	0	0	0	0	0	0
G. roseum	0	81.8	112.2	153.5	223.0	201.0
P. roqueforti	0	0	0	0	0	172.1
"Sterile mycelium"	0	55.7	132.5	181.0	215.0	224.5

The average dry-weight of the control mycelia grown in potato extract was 235.3 mg.

Here too the effect of the culture filtrate of *Penicillium expansum* was tested in higher dilutions, viz. in 1/64, 1/128, 1/256 and 1/512; the dry-weights proved to be 112.0 mg, 142.6 mg, 138.2 mg and 154.3 mg respectively. In comparing these results with those obtained with the culture on cherry juice it is clear that this antagonist produces a more active filtrate when it is grown in cherry juice than when it develops in potato extract; dilution of the filtrate of the cherry juice medium to 1/512 has about the same effect as dilution of the filtrate of the potato-extract medium to 1/64.

In the filtrates of the cultures of *Penicillium spiculisporum* in the potato-extract medium the development of *Thielaviopsis* was, just as in those of the cherry-juice cultures, of an abnormal type. The mycelium at the surface of the culture liquid was irregular, dark, slimy and watery, and showed clear indications of lysis. The control mycelia were at the surface of the medium dry and woolly, and showed a regular type of growth.

Noteworthy is the strong increase shown by the antagonistic activity of *Penicillium roqueforti*. With the other antagonists, with the exception of *Gliocladium roseum*, the production of antibiotics was in potato extract less than in cherry juice.

4.3. Production of antibiotics in a Czapek-Dox medium

In the previous experiments the antagonists were cultivated in crude plant extracts. It seemed worth while to investigate whether antibiotics would also be produced by these fungi when they were grown in a medium composed of chemically pure substances. So far investigators interested in the production of antibiotics have mostly made use of this kind of media, especially of the Czapek-Dox medium, to which occasionally stimulating substances were added (Birkinshaw and Raistrick, 1931; Wiesner, 1942; Waksman, Horning and Spencer, 1943; Katzman *et al.*, 1945; a.o.). For this reason it seemed appropriate to carry out a number of supplementary experiments with antagonists cultivated in this medium. The antagonists that were used for this series of experiments were *Penicillium roqueforti*, *Penicillium spiculisporum*, *Penicillium expansum*, *Aspergillus fumigatus* and *Penicillium spinulosum*. The method of filtering differed from that used in the preceding experiments in so far that instead of a Seitz filter a glas filter was used. An advantage of the latter is that it does not absorb antibiotics, which in the case of the Seitz filter is not entirely excluded (Van Luyk, 1938).

Each antagonist was inoculated in a series of 3 Erlenmeyer flasks with a capacity of 300 cc, each containing 50 cc of the Czapek-Dox medium. The inoculated flasks were kept for 17 days in an incubator at a temperature of 24° C, after which the culture liquid was filtered through filter-paper and subsequently sucked through a glas filter (17 G4). The cultures of *Penicillium spiculisporum* were kept in the incubator for 20 days, because this fungus grows rather slowly.

As preliminary experiments had shown that *Thielaviopsis basicola* does not grow in the Czapek-Dox medium, the culture filtrates of

the antagonists were mixed with cherry juice. This mixture was transferred by means of a pipette to sterilized test tubes which subsequently were inoculated with *Thielaviopsis*. For each dilution of the filtrate 5 tubes were used. Moreover, 3 series of controls were installed, viz. (1) with cherry juice, (2) with 3 parts of cherry juice to 1 part of Czapek-Dox, and (3) with 50 parts of cherry juice to 1 part of Czapek-Dox. The Czapek-Dox medium was added to the culture liquid of the controls because the culture liquid in the other tubes too consisted partly of this medium. The tubes were kept in the incubator for 8 days, after which the growth of the mycelium in the various tubes was compared; this was done at sight, and the results, expressed by means of the symbols 0, +, ++ and +++, are reproduced in Table 3. In the three series of control cultures the growth of *Thielaviopsis* was nearly the same.

TABLE 3

Growth of *Thielaviopsis basicola* in a diluted filtrate of the Czapek-Dox medium in which one of the listed antagonists had been cultivated, and to which subsequently cherry juice had been added

Antagonists	Degree of dilution of the filtrate							pH of undiluted filtrate
	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
P. roqueforti	+++	+++	+++	+++	+++	+++	+++	7.85
P. spiculisporum . . .	0	0	0	0	0	+	++	4.65
P. spinulosum	0	0	0	0	+	++	+++	5.90
P. expansum	0	0	0	0	0	++	+++	4.10
A. fumigatus	0	0	0	0	0	+	+	4.70

pH of the Czapek-Dox medium 6.15.

Explanation of symbols: 0 = no growth; + = very slight growth; ++ = growth about half as strong as that of the controls; +++ = approximately the same growth as in the controls.

As appears from Table 3, no or almost no antibiotics were formed in the Czapek-Dox medium by *Penicillium roqueforti*. With a dilution of the filtrate to 1/2 or 1/4 the growth of *Thielaviopsis* was even better than in the control cultures, but when the filtrate was applied in higher dilutions, the growth of the fungus decreased. It is, therefore, not impossible that *Penicillium roqueforti* produces substances which exercise a stimulating effect on the growth of *Thielaviopsis*.

In Table 4 the antagonistic activity of filtrates obtained from cultures of four of the antagonists in the Czapek-Dox medium is compared with that of filtrates obtained from cultures of the same antagonists in cherry juice and in potato extract. In this table, instead of expressing the dilution by means of fractions, use has been made of "dilution figures", e.g. instead of $^1/_{64}$ the figure 64. The "dilution figures" indicate the proportion between the volume of the diluted solution and the undiluted one from which it was prepared.

Table 4 shows that the antagonistic activity of *Penicillium spiculisporum* is approximately 16 times stronger when this fungus is grown in the Czapek-Dox medium than when it develops in cherry juice,

and approximately 32 times stronger than when it is cultivated in potato extract. The decrease in the amount of antibiotics produced in the two last-mentioned media, may be due perhaps to the presence in these crude plant extracts of substances which influence the metabolism of this fungus in such a way that a smaller amount of antibiotics is produced.

TABLE 4

Growth inhibition caused in *Thielaviopsis basicola* by filtrates of three different media in which four different antagonists had been cultivated. The inhibiting effect is expressed by means of two figures, the first indicating the dilution at which the growth of the fungus is still entirely suppressed, the second that at which growth just becomes possible

	A. fumigatus	*P. spinulosum*	*P. expansum*	*P. spiculisporum*
Czapek–Dox	64–128	32–64	64–128	64–128
Cherry juice	16– 32	4– 8	128–256	4– 8
Potato extract	8– 16	4– 8	32– 64	2– 4

The data of this table have been taken from the tables 1–3.

The antibiotic activity of *Aspergillus fumigatus* and of *Penicillium spinulosum* too appeared to be stronger when these fungi were grown in the Czapek-Dox medium. *Penicillium expansum*, on the other hand, developed its highest antibiotic activity when it was grown in cherry juice; in the Czapek-Dox medium its antibiotic activity was lower, and in the potato extract it was once more somewhat lower. Among the fungi that are not mentioned in Table 4, *Penicillium roqueforti* did not produce antibiotics when grown in the Czapek-Dox medium or in cherry juice, but it developed a strong antagonistic activity in the potato extract.

It appears therefore that the antagonistic activity of each of the fungi mentioned above has its own character, and that the demands they make on the medium in order to reach their highest output of antagonistic substances, differ for each of them.

4.4. Influence of the carbon source in the culture medium on the production of antibiotics

Fungi are heterotrophic organisms, which means that they are with regard to their carbon requirements dependent upon the organic compounds that are present in their environment. The nature of the carbon source and the amount in which it is available, will therefore play an important part in their metabolism, and indirectly these factors will exercise their influence on the activities of the fungi. For the production of antibiotics the importance of the carbon source has already been pointed out e.g. by Van Luyk (1938), Lochhead, Chase and Landerkin (1946) and Florey *et al.* (1949), and is now generally recognized. However, it seemed worth while to study this problem by using *Penicillium roqueforti* and *Penicillium spiculisporum*, because these two fungi were so far not known as anta-

gonists. This study concerns the influence exercised (a) by the nature of the carbon source, and (b) by its concentration.

4.4.1. *Nature of the carbon source*

In these experiments use is made of a synthetic culture solution which as to its inorganic constituents agreed with a Czapek medium. This inorganic part of the culture liquid was pipetted into a number of Erlenmeyer flasks with a capacity of 500 cc; each of these flasks subsequently received a different carbohydrate as carbon source. These carbohydrates were glucose, maltose, saccharose, galactose, lactose, soluble starch, dextrin, fructose, maltose + casein, and brown sugar. In this way ten different culture media were obtained which differed only in the nature of the carbon source. In each of these media the carbon source constituted 3 % of the total; in the case in which the carbon source consisted of maltose mixed with casein 2 % was formed by the maltose and 1 % by the casein.

For each medium 4 Erlenmeyer flasks were used, and each flask received 50 cc of the medium. After being sterilized they were inoculated with one of the fungi.

The first set of experiments was carried out with *Penicillium roqueforti*. The flasks that had been inoculated with this fungus were kept for 17 days in an incubator at a temperature of 24° C.

Although there appeared to be much difference in the way in which *Penicillium roqueforti* developed in the various media, there was only one medium, viz. that containing lactose, which proved to be unsuitable for the cultivation of this fungus. At the end of the incubation period only a very slight growth was found in this medium, and for this reason these cultures had to be discarded. In the medium containing saccharose it showed a luxuriant but irregular growth, the surface of the mycelium being wavy, blotched with green patches and covered with droplets of a yellowish-brown liquid. In the media containing soluble starch the mycelium needed nearly a week to develop the green colour which in the media containing other carbohydrates developed almost immediately; however, at the end of this period the surface proved to be uniformly green; the exudation of droplets was here very strong. This exudation occurred also in the other media. The colour which the culture liquid finally assumed, differed considerably, viz. from light yellow to dark red.

At the end of the period of 17 days during which the cultures were kept in the incubator, the culture liquid of the various flasks was filtered in the usual manner, and just as in the preceding experiments various dilutions were prepared by mixing the filtrate with sterilized cherry juice. These dilutions were transferred to sterilized test tubes, 5 tubes being used for each dilution, and just as in the preceding series of experiments three sets of controls were instituted; in one set the medium consisted of cherry juice alone, in the two other sets of cherry juice mixed with a different proportion of the synthetic medium. The tubes were inoculated in the usual way with *Thielaviopsis*. After 8 days the growth of the latter in the various sets

was compared. The results are given in Table 5, in which the same symbols have been used as in Table 3. Here too in the three sets of controls the growth appeared to be nearly the same.

TABLE 5

Growth of *Thielaviopsis basicola* in culture filtrates obtained from cultures of *Penicillium roqueforti* in media differing in the nature of the carbon source; the filtrates were diluted with cherry juice

Carbon source	Degree of dilution of the filtrate						pH of medium	pH of undiluted filtrate
	1/2	1/4	1/8	1/16	1/32	1/64		
1. galactose . . .	0	+	+++	+++	+++	+++	6.00	7.90
2. fructose . . .	0	0	0	++	+++	+++	5.50	6.60
3. glucose . . .	+++	+++	+++	+++	+++	+++	5.80	8.65
4. saccharose . .	0	0	0	0	0	0	6.10	6.05
5. brown sugar .	0	0	0	0	+	++	6.00	6.85
6. maltose . . .	0	0	0	+	++	+++	5.80	7.10
7. soluble starch .	+	+++	+++	+++	+++	+++	6.35	8.55
8. dextrin	+++	+++	+++	+++	+++	+++	6.10	8.70
9. maltose + casein	+++	+++	+++	+++	+++	+++	5.50	8.75

Explanation of symbols, see Table 3.

It appears from Table 5 that the nature of the carbon source exercises a marked influence on the production of antibiotics by *Penicillium roqueforti*. In the medium containing saccharose this fungus developed by far its highest antagonistic activity. Even in the highest dilution of the filtrate that was tested in this series of experiments, viz. 1/64, the growth of *Thielaviopsis* proved to be completely suppressed. Afterwards the experiment was repeated with a dilution of $^{1}/_{128}$, and then a very slight growth was observed.

In the media containing monosaccharides, viz. glucose, galactose and fructose, as well as in those containing the disaccharides saccharose, maltose and brown sugar the antagonistic activity proved to differ.

The fact that in the medium containing glucose no growth-inhibiting substances were produced, whereas in the one containing fructose a fair amount, and in that with saccharose by far the largest quantity of these substances was produced, is doubtless unexpected. With regard to saccharose two possibilities should be considered, viz. that this substance is assimilated directly or that it is first hydrolysed. As in the latter case a mixture of equal parts of glucose and fructose would result, the fact that the antibiotic activity in the medium containing saccharose surpasses that in the medium containing fructose, could be explained only by assuming that the presence of one of these substances stimulates the influence exercised by the other. That the presence of one sugar may influence the utilization of another one, was shown by Horr (1936). Lilly and Barnett (1953) too point out that "the behaviour of fungi in the presence of mixed sugars is not always predictable from their behaviour on the single sugars comprising the mixture." However, there

is still an entirely different explanation, viz. that the three sugars (glucose, fructose and saccharose) undergo changes during the autoclaving (Barnett, Lilly and Waters, 1953). Lilly and Barnett state that "fructose darkened more than glucose when autoclaved. It is presumed that fructose is altered more by this method of sterilization than are the aldo sugars". Preliminary experiments of these investigators indicated that an inhibitory substance(s) is formed when fructose is autoclaved with the other constituents of the medium.

Another remarkable fact is that in the medium containing a mixture of maltose and casein as carbon source, no antiobiotics were produced, whereas in a medium in which the carbon source consisted of maltose alone, there were clear indications that a production of such substances actually took place. It seems plausible to assume that *Penicillium roqueforti* is influenced by the presence of casein in such a way that it produces little or no antiobiotics. That in the medium which contained brown sugar, a smaller amount of growth-inhibiting substance was produced than in one containing pure saccharose, may be due to the presence in the brown sugar of substances with a similar effect.

A similar set of experiments was performed with *Penicillium spiculisporum*. This fungus was cultivated in Czapek media with different carbon sources, the incubation period being 20 days. The substances used as carbon source were the same as in the experiments with *Penicillium roqueforti*, and they were used in the same concentration. The best growth was observed in the media containing glucose, maltose, saccharose, galactose and brown sugar. In the medium containing lactose growth was so insignificant that it did not seem worth while to prepare a filtrate. In the remaining media the development was fair, although much slower than in the first-mentioned ones.

The fact that *Penicillium roqueforti* as well as *Penicillium spiculisporum* show with lactose as carbon source but a very weak growth, whereas they grow well with glucose or with galactose, i.e. with the two monosaccharides that are formed when lactose is hydrolysed, is doubtless due to the circumstance that these two fungi are unable to hydrolyse this disaccharide to an appreciable extent. Lilly and Barnett (1953) also arrived at the conclusion that "lactose is a poor source of carbon for fungi." They cultivated a number of fungi, one of which was *Penicillium spiculisporum*, on a medium containing lactose as carbon source as well as on one containing the two sugars that are formed when lactose is hydrolysed, and concluded from their experiments that "fungi failing to grow on lactose are unable to hydrolyse this sugar."

The culture filtrates of *Penicillium spiculisporum* were almost colourless, except with the medium containing brown sugar and in that with fructose, where the colour was light yellow. In order to test their antibiotic activity on *Thielaviopsis* they were diluted with sterilized cherry juice. The results of the experiments are summarized in Table 6.

In these experiments the culture filtrates of *Penicillium spiculisporum* developed a much stronger antibiotic activity than those of *Penicillium roqueforti* did in the preceding set, stronger, moreover, than would have been expected. In two instances, viz. in the filtrates of the media containing glucose and maltose, even the highest dilution that was

TABLE 6

Growth of *Thielviopsis basicola* in filtrates obtained from cultures of *Penicillium spiculisporum* in Czapek media differing in the nature of the carbon source; the filtrates were diluted with cherry juice

Carbon source	Degree of dilution of the filtrate						pH of undiluted filtrate
	1/8	1/16	1/32	1/64	1/128	1/256	
1. galactose	0	0	0	+	++	+++	6.10
2. glucose	0	0	0	0	0	0	4.60
3. fructose	0	0	0	+	++	+++	5.05
4. saccharose	0	0	0	0	+	++	4.70
5. brown sugar	0	0	0	0	0	+	4.75
6. maltose	0	0	0	0	0	0	4.60
7. soluble starch	0	0	0	0	+	++	5.80
8. dextrin	0	0	0	0	0	+	4.70

Explanation of symbols, see Table 3.

tested, viz. 1/256, caused a complete inhibition of the growth of *Thielaviopsis*. Afterwards the filtrates of these cultures were tested also in higher dilutions. In the dilution 1/512, the filtrate from the maltose culture proved to allow about half the normal growth, and in the dilution 1/1024 a normal one, whereas by the filtrate from the glucose culture in the dilution 1/512 still all growth was inhibited, and in the dilution 1/1024 half the normal growth was allowed.

Although the amounts of antibiotics that were produced by *Penicillium spiculisporum* in media with different carbon source, proved to differ, in all the media that were used in this set of experiments, such substances nevertheless were formed, and in this respect the results of these experiments differ therefore from those that were obtained in the experiments with *Penicillium roqueforti* (Table 5), in which no antibiotics were found in the filtrates of cultures containing glucose, dextrin or the mixture of maltose and casein. Other noteworthy differences are that *Penicillium spiculisporum* develops in the medium containing brown sugar a stronger antibiotic activity than it does in the medium containing pure saccharose, and that the proportion between the amounts produced in the medium containing saccharose and in the media which contained the substances that arise when the latter is hydrolysed, was as might be expected when we assume (1) that the saccharose is hydrolysed before it is assimilated and (2) that the products of the hydrolyzation do not influence the utilization of each other.

4.4.2. *Concentration of the carbon source*

The experiments of which the results were summarized in Table 5, have shown that in the case of *Penicillium roqueforti* saccharose is the

most suitable carbon source for the production of antibiotics. In these experiments the culture medium contained 3 % saccharose, but as it seemed possible that a change in the concentration of the saccharose might give different results, a number of experiments have been performed in which this concentration was varied.

In the same way as in the earlier experiments with *Penicillium roqueforti* here too a medium was used which contained the same inorganic constituents as the Czapek one. With this medium 5 Erlenmeyer flasks were filled, each flask receiving 200 cc and in addition a definite weight of saccharose; in this way saccharose concentrations were obtained of 1 %, 2 %, 3 %, 4 % and 5 %. The pH of the media proved to vary between 6.05 and 6.10. Each of these liquids was distributed over 4 Erlenmeyer flasks, so that each of the latter received 50 cc. After sterilization the flasks were inoculated with *Penicillium roqueforti*.

The growth of the fungus differed but slightly in the various media; in that with 1 % saccharose, however, it remained behind. The exudation of droplets was strong in the media with 1 %, 2 % and 3 % saccharose, but less strong in those with 4 % and 5 %; in the medium with 1 % saccharose they were colourless, and in those with 2 % and 3 % of a characteristic chestnut-brown.

After an incubation period of 17 days the culture medium was filtered. The filtrate of the medium with 1 % saccharose proved to be yellow-brown, that of the other ones cherry-red. After dilution with cherry juice the antibiotic activity of the filtrate was tested in the usual way on *Thielaviopsis* (Table 7).

TABLE 7

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* in Czapek media containing from 1 % to 5 % saccharose; the filtrates were diluted with cherry juice

Concentration of saccharose	Degree of dilution of the filtrate					pH of undiluted fiiltrate
	1/16	1/32	1/64	1/128	1/256	
1 %	+++	+++	+++	+++	+++	8.10
2 %	0	++	+++	+++	+++	7.95
3 %	0	0	0	++	+++	6.45
4 %	0	0	0	+	++	5.85
5 %	0	0	0	0	++	5.90

Explanation of symbols, see table 3.

In these experiments the antagonistic activity of the filtrate appeared to increase regularly with the increase of the sugar concentration in the culture medium. The experiments confirmed the conclusion reached in the earlier ones according to which saccharose is to be regarded as a carbon source which allows this fungus to develop a strong antagonistic activity; they also confirmed the impression created by the earlier experiments that the development of a strong antagonistic activity is accompanied by a comparatively low pH.

The results obtained by cultivating the antagonist in media con-

taining saccharose, however, are not always the same. In the next section some experiments will be described which prove that, judged by the antagonistic activity of the culture filtrate, the utilization of the saccharose may be severely hampered by the presence of other organic compounds in the medium.

4.5. Comparison of the antagonistic activity of filtrates obtained from cultures of Penicillium roqueforti in potato extract and in a Czapek medium

The experiments with *Penicillium roqueforti* in media which differed in the nature of the carbon source, have shown that this fungus does not produce antibiotics in media in which glucose is the only carbon source, whereas in media containing saccharose as the only carbon source, the production of antibiotics is highest (Table 5). Earlier in this work it appeared, however, that a high antagonistic activity was also developed when the fungus was cultivated in potato extract to which 2 % glucose had been added (Table 2). In a medium with soluble starch as the only source of carbon, on the other hand, hardly any antagonistic activity was developed, so that the strong antagonistic activity reached in the potato extract can not be due, at least not directly, to the starch that is present in the latter. It looks therefore as if the potato extract contains one or more other substances which affect the production of antibiotics. In order to study the effect of such substances on the utilization of glucose and saccharose a set of experiments were carried out in which *Penicillium roqueforti* was cultivated in potato extract and in a Czapek medium to which glucose or saccharose had been added. In these experiments 5 media were used of which the composition is given in Table 8. With each medium three cultures were made, and at the end of 17 days all the cultures were filtered, and the filtrates diluted with various amounts of potato extract. The antagonistic activity of these dilutions was tested in the usual way on *Thielaviopsis*. The results are given in table 8.

It appears that the antagonistic activity decreased in a Czapek

TABLE 8

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* in various media; the filtrates were diluted with potato extract

Culture medium of *Penicillium roqueforti*	Degree of dilution of the filtrate					pH of undiluted filtrate
	1/8	1/16	1/32	1/64	1/128	
1. Czapek medium + 3 % glucose	+++	+++	+++	+++	+++	8.15
2. Potato extract + 3 % glucose	0	0	+	++	+++	5.90
3. Czapek medium + 3 % saccharose	0	0	0	0	++	5.93
4. Potato extract + 3 % saccharose	+++	+++	+++	+++	+++	6.50
5. A mixture of 3 and 4	+++	+++	+++	+++	+++	7.25

Explanation of symbols, see table 3.

medium with glucose and in a potato extract to which saccharose had been added, and that it increased in a Czapek medium with saccharose and in a potato extract with glucose. The filtrate of a mixture of the two media containing saccharose had no antagonistic activity.

LILLY and BARNETT (1953) arrived at the conclusion that "the utilization of sugars (either alone or in mixtures) is modified by a number of factors, which include other constituents of the medium, the environment and time."

4.6. INFLUENCE OF STIMULANTS ON THE PRODUCTION OF ANTIBIOTICS

That some substances may exercise a stimulating influence on the activities of micro-organisms has already been known for a considerable time, and that the production of antiobiotics too may be affected by such substances has been shown by several investigators. KATZMAN *et al.* (1944) found that the production of antibiotics by *Aspergillus clavatus* is favoured by "corn steep liquor"; they recommend to add 2 cc of this substance to one liter of the culture medium. LOCHHEAD, CHASE and LANDERKIN (1946), on the other hand, found that the production of antibiotics by two different species of *Penicillium* was depressed by the presence of corn steep liquor. MOYER and COGHILL (1946a), however, report that the production of penicillin in a Czapek-Dox medium is increased 6 to 8 times when so much corn steep liquor is added that the medium consists for 7.5 % to 10 % of this extract.

In the next set of experiments the influence of commercial corn steep liquor was studied once more. The experiments were made with *Penicillium roqueforti*, and different concentrations of the corn steep liquor were tried.

Five Erlenmeyer flasks received each 200 cc of a Czapek medium with 5 % saccharose. To four of these flasks amounts of 2, 4, 8 and 12 cc corn steep liquor were added; the culture liquid in these flasks contained therefore respectively 1 %, 2 %, 4 % and 6 % of this extract; the fifth flask contained no corn steep liquor and served as a control. The contents of each flask were divided over 4 other flasks, and after sterilization the latter were inoculated with *Penicillium roqueforti*.

The fungus developed more luxuriously and far more rapidly in the media which contained the corn steep liquor than in the control flasks. When in the latter the first signs of growth became discernible, there was already a closed layer of mycelium at the surface of the medium in the other flasks. In the beginning this fleece had a light green colour, but in the media containing the higher concentrations of the corn steep liquor it afterwards became brown. When the cultures were 15 days old, there appeared to be a distinct gradation from the green colour in the cultures containing 1 % corn steep liquor to the brown one of the cultures which contained 6 % of this admixture. In the media containing this stimulating agent the myce-

lium is evenly coloured, whereas it shows patches of white in the controls. In the media containing corn steep liquor it forms, moreover, a thick and solid, strongly wrinkled fleece; and the thickness and solidity of the latter increases with the concentration of the corn steep liquor. Furthermore, the exudation of droplets is more abundant in the media with the corn steep liquor than in the controls.

At the end of a period of 15 days the culture liquid was filtered, and the filtrate diluted in the usual way with cherry juice. The antibiotic activity of these dilutions was tested by the aid of *Thielaviopsis* (Table 9).

TABLE 9

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* in a Czapek medium containing varying concentrations of corn steep liquor; the filtrates were diluted with cherry juice

Percentage of corn steep liquor	Degree of dilution of the filtrate					pH of medium	pH of undiluted filtrate
	1/32	1/64	1/128	1/256	1/512		
0	0	0	+	++	+++	6.30	6.75
1	0	+	++	++	+++	4.90	6.77
2	+++	+++	+++	+++	+++	4.70	7.76
4	+++	+++	+++	+++	+++	4.60	8.60
6	+++	+++	+++	+++	+++	4.50	8.60

Explanation of symbols, see Table 3.

Table 9 shows that the production of antibiotics underwent, in conformity with the findings of Lockhead, Chase and Landerkin (1946), a marked decrease in the media containing an admixture of corn steep liquor. Unfortunately we did not test the effect of concentrations higher than that of the dilution 1/32, but it seems nevertheless justified to assume that the growth-inhibiting effect of the filtrates decreases with an increase of the concentration of the corn steep liquor in the culture medium.

4.7. Influence of the incubation time on the production of antibiotics by Penicillium roqueforti

In the experiments that so far have been described, the incubation period of *Penicillium roqueforti* always lasted from 15 to 20 days. However, it is not unthinkable that the amount of antibiotics which is produced in a given period, may show some relation to the rate of growth, and as the latter decreases after some time, the values found in these experiments do not necessarily give an entirely satisfactory picture of the production process. To test this possibility some experiments were made in which the incubation time was varied.

These experiments were performed with 18 Erlenmeyer flasks of 300 cc capacity, each containing 50 cc of a Czapek medium with 5 % saccharose as carbon source. This group of 6 times 3 flasks were inoculated with *Penicillium roqueforti*, and placed in an incubator at a temperature of 24° C. After 10 days the first group of 3 flasks were taken out of the incubator, and then with intervals of 5 days the other

groups were removed, the contents filtered, and the filtrates tested in the usual way by estimating their effect on the growth of *Thielaviopsis* (Table 10).

It seems that *Penicillium roqueforti* completes its growth under the circumstances of the experiment in about 10 days; in the subsequent period little or no further expansion was observed. At the end of these 10 days the culture filtrates appeared to have assumed already the cherry-red colour which is characteristic for this strain of the fungus, and the exudation of droplets had just started.

TABLE 10

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of *Penicillium roqueforti* of different age; the cultures were made in a Czapek medium containing 5 % saccharose, and the filtrates were diluted with cherry juice

Incubation time in days	Degree of dilution of the filtrate						pH of undiluted filtrate
	1/16	1/32	1/64	1/128	1/256	1/512	
10	0	0	0	+	++	+++	7.15
15	0	0	0	0	+	+++	5.90
20	0	0	0	0	+	+++	5.50
25	0	0	0	0	+	+++	5.40
30	0	0	0	0	+	+++	5.20
35	0	0	0	+	++	++	5.20

pH of the Czapek medium 6.20.
Explanation of symbols, see Table 3.

It appears from table 10 that *Penicillium roqueforti* needs about 15 days to produce the maximum amount of antibiotics; when the cultivation is continued for a longer period, no more antibiotics or at least no appreciable amounts of antibiotics are produced. It seems therefore that this fungus produces its antibiotics during its period of active growth. When the cultures were left in the incubator for 35 days, the amount of antibiotics in the culture liquid seemed to decrease. This decrease can not be due to instability of the antibiotics, as the latter appear to be comparatively stable (see Table 11). It seems more probable that the antibiotic activity decreases because of the presence of other metabolites in the culture medium or else because part of the antibiotics is absorbed by the fungus itself.

From various experiments the impression was gained that the production of antibiotics by the strain of *Penicillium roqueforti* that was used here, is remarkably constant, at least so long as the external conditions remain the same. With other antagonists this is not always so. With *Penicillium expansum*, for instance, VAN LUYK (1938) noted a considerable degree of variability in the production of antibiotics.

4.8. STABILITY OF THE ANTIBIOTICS IN THE CULTURE FILTRATES

Several of the antibiotics are apparently not very stable, for the filtrates in which they are present, tend to lose their antagonistic activity to some extent, for instance when they are exposed to higher temperatures or simply when they are stored for some time (WEIND-

LING and EMERSON, 1936; VAN LUYK, 1938; WEINDLING, 1941; BRIAN and McGOWAN, 1945; BRIAN, CURTIS, HEMMING and McGOWAN, 1946; and others).

It seemed worth while to find out whether the antibiotics in the culture filtrates of *Penicillium expansum*, *Penicillium spinulosum*, *Aspergillus fumigatus*, *Penicillium spiculisporum*, *Penicillium roqueforti* and *Gliocladium roseum* are able to withstand a 10-minutes heat-sterilization at 103° C.

The antagonists were cultivated for 16 days in a Czapek-Dox medium at a temperature of 24° C, but as *Penicillium roqueforti* does not produce antibiotics in this medium, for this species a Czapek medium was used to which 5 % saccharose had been added, and as *Penicillium spiculisporum* is a slow grower, the latter was kept three days longer in the incubator. At the end of the incubation period the culture media were filtered through filter-paper, and the filtrates divided in two parts; one part was placed in an autoclave and exposed for 10 minutes to a temperature of 103° C, whereas the other part was sucked through a glass filter. Of both parts dilutions were made with cherry juice, and the antagonistic activity of the filtrates were tested in the usual way on *Thielaviopsis*. Cultures of this fungus on cherry juice without an admixture of filtrate were used as controls.

It appeared that the filtrates of *Aspergillus fumigatus* and of *Gliocladium roseum* had lost in the autoclave about half their antagonistic activity, whereas the antibiotic activity of the other filtrates showed no decrease.

The possibility that the antibiotic activity of the culture filtrates might decrease in the course of time, has also been investigated. In these experiments the same antagonists were used as in the preceding ones with the exception of *Gliocladium roseum*, and here too they were cultivated in a Czapek-Dox medium to which in the case of *Penicillium roqueforti* 5 % saccharose had been added. The incubation time and the incubation temperature too were the same.

The culture filtrates and the dilutions of the latter with cherry juice were prepared in the usual way, and each dilution was divided over four parallel series. One of the latter was inoculated immediately with *Thielaviopsis*, wheares the three other ones were stored, together with controls, at room temperature. The second, third and fourth series were inoculated respectively on the 16th, the 31st and the 46th day. The results of the tests are recorded in Table 11.

On the whole the culture filtrates retained their antibiotic activity in the period of 45 days during which they were stored. In that of *Aspergillus fumigatus*, however, it decreased in 30 days to about half its original value.

In the filtrate of *Penicillium roqueforti* that had been diluted to 1/128, after 15 days storage only a slight growth of *Thielaviopsis* was noticeable, and in the filtrate that had been diluted to 1/256, after the same period of storage growth reached about half the normal rate. It looks, therefore, as if the antibiotic activity of the filtrate had increased. In the filtrates that had been stored for 30 and 45 days, the growth of *Thielaviopsis* seemed to have decreased even somewhat

further. It is not impossible that this increase of the antibiotic activity of the filtrate may be due to a loss of water by evaporation. i.e. to an increase in the concentration of the solution. That it would be accidental, i.e. a result of the variability of *Thielaviopsis*, is hardly believable.

TABLE 11

Growth of *Thielaviopsis basicola* in filtrates obtained from cultures of various antagonists and stored for a different length of time; the filtrates were diluted with cherry juice

Name of antagonist and pH of filtrate	Storage in days	Degree of dilution of the filtrate 1/32	1/64	1/128	1/256	1/512
Penicillium expansum 4.10	0	0	0	++	+++	+++
	15	0	+	++	+++	+++
	30	0	+	++	+++	+++
	45	0	+	++	+++	+++
Penicillium spinulosum 5.90	0	0	+	++	+++	+++
	15	0	+	++	+++	+++
	30	0	+	++	+++	+++
	45	0	+	++	+++	+++
Aspergillus fumigatus 4.70	0	0	0	+	+	++
	15	0	0	+	+	++
	30	0	+	+	++	+++
	45	0	+	+	++	+++
Penicillium spiculisporum 4.65	0	0	0	+	++	+++
	15	0	0	+	++	+++
	30	0	0	+	++	+++
	45	0	0	+	++	+++
Penicillium roqueforti 5.75	0	0	0	++	+++	+++
	15	0	0	+	++	+++
	30	0	0	+	++	+++
	45	0	0	+	++	+++

Explanation of symbols, see Table 3.

The loss of water by evaporation was estimated by comparing in the test tubes which contained the diluted filtrates, the average height of the liquid column at the beginning of the experiment and 30 days later. It appeared that the loss amounted to approximately 1/8 of the original volume. This loss might have been compensated by the addition of a corresponding volume of water, but this was not done because in that case the results obtained with this series would not have been fully comparable to those obtained with the other ones. At any rate, if this loss would explain the increase of the antibiotic activity, this increase would be spurious, and then the antibiotic activity of the culture filtrate of this fungus would have to be regarded as remarkably constant.

4.9. Influence exercised on the growth of Thielaviopsis basicola by culture filtrates mixed with an agar medium

So far the effect exercised by the culture filtrates of the antagonists

on *Thielaviopsis* was tested only in liquid media. A disadvantage of this method is that the effect can be determined only once, viz. at the end of the experiment, and not in the course of the latter. If the filtrates are dissolved in a molten agar medium, the growth of the *Thielaviopsis* colony which develops on the congealed agar, can be measured at various intervals, and in this way eventually the presence of changes in the antibiotic activity of the filtrates may be detected. It might be objected that the antibiotics are perhaps partly absorbed by the agar, a possibility that was already suggested by Van Luyk (1938). Slagg and Fellows (1947), however, cultivated a large number of soil fungi on media of this kind in order to estimate the antibiotic activity of the latter, and found the method most convenient.

The following set of experiments were carried out in order to study the behaviour of *Thielaviopsis* on agar media containing antibiotics. In order to obtain such a medium the antagonists were cultivated in cherry juice at a temperature of 24° C, and after 16 days the culture liquid was filtered in the usual way. Tubes containing 15 cc cherry-agar were sterilized and placed in a water bath at a temperature of 45° C, and then to each of the tubes 5 cc undiluted or diluted filtrate was added; in this way a series of dilutions ranging from 1/4 to 1/128 were obtained. The culture filtrate of *Aspergillus fumigatus* was further diluted to 1/256, and that of *Penicillium expansum* to 1/512. The contents of the tubes were transferred to Petri dishes and inoculated with *Thielaviopsis*. The inoculation was carried out by means of round discs of mycelium with a diameter of 3 mm. At the same time a series of controls was instituted which consisted of 15 cultures; the medium of the latter was prepared by adding instead of filtrate 5 cc sterilized cherry juice to the molten agar. The cultures were placed in an incubator and kept at a temperature of 24° C, and from time to time the linear growth of each colony was estimated. To this end the diameter of the colony was measured in two directions, one perpendicular to the other. From the diameter 3 mm, i.e. the diameter of the disc that was used for the inoculation, was subtracted.

The effect of each dilution was tested in five cultures, and in Table 12 the average diameter (minus 3 mm) reached by these colonies in the first five days and their subsequent increase in three periods of three days are recorded.

Table 12 shows that the increase in diameter of the *Thielaviopsis* colonies in the three consecutive periods of three days was approximately the same. It can certainly not be denied that the rate of growth fluctuated somewhat, e.g. in the tests with the culture filtrate of *Aspergillus fumigatus*, but there is no regularity in these fluctuations, and they may therefore probably be ascribed to accidental circumstances. In the tests with the culture filtrate of *Penicillium spinulosum*, however, the rate of growth appeared to increase fairly regularly. This phenomenon remains for the moment inexplicable; it might mean that *Thielaviopsis* is able to adapt itself to some extent to the presence of this filtrate.

On the whole *Thielaviopsis* showed itself very sensitive with regard

TABLE 12

Increase in diameter in mm shown by colonies of *Thielaviopsis basicola* cultivated on cherry-agar to which culture filtrates of antagonists had been added in various dilutions

Name of antagonist and pH of filtrate	Dilution of filtrate	Diameter reached in 5 days	Increase in diameter in 3 periods of 3 days			Average increase per period	Same as % of normal increase
			1	2	3		
Penicillium	1/4	0	0	0	0	0	0
spiculisporum	1/8	5.0	3.5	3.2	2.8	3.2	27
5.20	1/16	8.5	2.5	2.5	2.5	2.5	21
	1/32	11 0	7 5	7.5	7.2	7.4	63
	1/64	12.0	8.0	7.5	8.0	7.8	66
	1/128	13.0	9.0	8.0	8.5	8.5	72
	Controls	16.0	12.0	11.7	11.5	11.7	100
Penicillium	1/4	0	0	0	0	0	0
spinulosum	1/8	0	0	0	0	0	0
4.80	1/16	0	2.0	2.8	5.4	3.4	30
	1/32	1.5	2.0	2.5	5.0	3.2	28
	1/64	4.2	6.3	7.2	7.0	6.8	60
	1/128	9.4	7.8	8.3	9.2	8.4	74
	Controls	16.0	11.0	12.0	11.0	11.3	100
Aspergillus	1/16	0	0	0	0	0	0
fumigatus	1/32	0	1.7	1.5	0.8	1.3	12
5.30	1/64	1.0	2.0	1.5	1.9	1.8	16
	1/128	3.3	4.7	4.0	5.2	4.6	41
	1/256	5.6	6.6	6.8	8.5	7.3	65
	Controls	16.0	11.0	12.0	11.2	11.4	100
Penicillium	1/16	0	0	0	0	0	0
expansum	1/32	0	0	0	0	0	0
4.20	1/64	0	0	2.0	2.5	2.3	20
	1/128	4.0	7.0	7.0	7.4	7.1	63
	1/256	8.0	8.5	8.5	9.0	8.7	77
	1/512	12.0	9.5	9.7	10.1	9.8	87
	Controls	16.0	11.0	12.0	11.0	11.3	100
Penicillium	1/4	6.5	7.0	8.0	8.5	7.8	69
roqueforti	1/8	10.0	9.2	9.8	10.0	9.7	86
5.50	1/16	12.5	10.5	11.0	11.0	10.8	96
	1/32	14.0	11.6	10.4	11.5	11.2	99
	1/64	15.0	11.6	11.6	11.8	11.7	103½
	1/128	15.0	11.5	11.7	11.8	11.7	103½
	Controls	16.0	11.0	12.0	11.0	11.3	100

to the antibiotics that were tested in these experiments. With an increasing dilution of the filtrate, the rate of growth of the test fungus gradually increased. The conclusion therefore seems justified that a solid culture medium may be regarded as a very suitable substrate for testing the antibiotic activity of culture filtrates.

It can, on the other hand, not be denied that *Thielaviopsis* sometimes showed deviations from its normal development. On media to which the to 1/8 or to 1/16 diluted culture filtrate of *Penicillium spiculisporum* had been added, the mycelium showed an abnormal aspect, and even

lysis of the hyphae, and the culture filtrates of *Penicillium spinulosum* and of *Aspergillus fumigatus* caused the development of so many white sectors in the colonies that the latter looked decidedly blotched. However, as the white sectors appeared to expand in the same way as the green ones, their presence will not have affected the results of the growth measurements.

When the figures for the increase in the test cultures expressed as a percentage of the increase in the control cultures are compared with each other, it appears that the culture filtrate of *Penicillium expansum* had the strongest antibiotic effect. Even in a dilution of 1/32 it completely inhibited the growth of *Thielaviopsis*. With the culture filtrates of *Aspergillus fumigatus*, *Penicillium spinulosum* and *Penicillium spiculisporum* a total growth inhibition required a concentration corresponding to a dilution respectively to 1/16, 1/8 and 1/4. With the culture filtrates of *Penicillium expansum* at a dilution to 1/128 a growth corresponding to 63 % of that found in the control cultures was reached, whereas with the culture filtrates of *Aspergillus fumigatus* a similar growth required a dilution to 1/256, with those of *Penicillium spinulosum* a dilution to 1/64, and with that of *Penicillium spiculisporum* a dilution to 1/32. The culture filtrate of *Penicillium roqueforti* exercised but a weak antibiotic action; with a dilution to 1/4 the growth of *Thielaviopsis* still amounted to 69 % of the value found in the control cultures, whereas at a dilution of 1/32 the difference between the test culture and the control had entirely disappeared.

4.10. Production of antibiotics by various strains of Penicillium roqueforti

Although the various strains belonging to the same fungus species show, as a rule, little or no difference in aspect, there is sometimes a considerable difference in behaviour. Such a difference is recognizable, for instance, in the tables 1 and 2 in the production of antibiotics by two strains of *Aspergillus fumigatus*, and similar examples have frequently been given in the literature (Van Luyk, 1938; Jaarsveld, 1942; a.o.).

In the following series of experiments the production of antibiotics by the strain of *Penicillium roqueforti* that so far had been used in the experiments, was compared with that of five strains belonging to the culture collection of the "Central Bureau of Fungus Cultures (C.B.S.)" at Baarn (Netherlands). These five strains had been incorporated in the collection in the years 1929, 1930 and 1938. A strain isolated from French roquefort cheese was tested too.

Of each strain three cultures were made in a Czapek medium containing 3 % saccharose, and at the end of an incubation period of 17 days the culture liquid was filtered, and the antibiotic activity of the filtrate in dilutions starting with 1/4 tested on *Thielaviopsis*.

The filtrate obtained from the strain that in the course of this investigation was isolated from soil, caused at a dilution of 1/64 a complete inhibition of the growth of *Thielaviopsis*, whereas at a

dilution of 1/128 a slight growth of the test fungus was noticeable. The filtrate of one of the strains received from the "C.B.S." allowed a slight growth at a dilution of 1/4 and normal growth at one of 1/8. The filtrates of the remaining strains caused no inhibition at all. In the filtrates of three of the latter the growth of the test fungus was better in the less diluted than in the more diluted filtrates and even better than in the control cultures.

These experiments on the production of antibiotics by various strains, therefore, have clearly shown how considerably the strains may differ in this respect. The first-investigated strain is a good producer of antibiotics, whereas the other ones are in this respect of little or no importance.

4.11. The way in which different strains of thielaviopsis basicola react on antibiotics

The preceding section dealt with the way in which different strains of the same fungus species may differ in their production of antibiotics. Here the way in which different strains of the same test fungus react on the presence of these antibiotics, will be considered. That these strains may differ in this respect, was known already. Stover (1950a) found that the two "cultural types" of *Thielaviopsis basicola* which were isolated by him from tobacco, differed in their reaction on the presence of a bacterial antagonist indicated as "A". The brown "cultural type" proved to be strongly inhibited, while the mycelium of the grey one became submerged but its growth was not greatly retarded.

In order to find out whether there are differences in the way in which various strains of *Thielaviopsis basicola* react on the culture filtrate of *Penicillium roqueforti*, six of them were tested, of which 4 were obtained from the "C.B.S.". In cultures on cherry-agar these strains appeared to show different properties.

(1) The strain that was isolated by Mooi–Bok in 1949 from *Lathyrus odoratus*, grows slowly and stops growing when but a part of the agar surface is covered. The cultures are green at the bottom and white with much aerial hyphae at the upper side. Spores are produced but scarcely and often not at all.

(2) The strain that was isolated by Tiddens in 1933 from Poinsettia (*Euphorbia pulcherrima*), grows luxuriously with much aerial mycelium. The cultures are green, dark green to nearly black at the bottom and greyish at the upper side. In comparison with the luxurious production of mycelium the production of spores is but scanty.

(3) This strain was received from America where it was isolated by Gilbert from tobacco; it is since 1926 in the collection of the "C.B.S.". The properties of the cultures agree with those observed in the strain that was isolated from Poinsettia. Its growth is luxurious with much aerial mycelium, but the colour of the cultures is darker, and more spores are produced. There is a tendency to form sectors.

(4) The cultures of this strain, which was isolated in 1948 by Van Holder from *Cypripedium* roots, remain appressed to the agar

substrate and develop less aerial mycelium. At the bottom the cultures are dark green and at the upper side light green. A large amount of endoconidia and of chlamydospores are produced.

(5) The strain that was isolated from the roots of *Primula obconica* grows slowly, and the mycelium remains appressed to the agar substrate and produces but few aerial hyphae. The cultures are dark green at the bottom and green at the upper side. Endocondidia and chlamydospores are produced in very large numbers. There is a tendency to produce sectors.

(6) The strain that was isolated from the roots of *Nicotiana glutinosa* grows rapidly and produces much aerial mycelium, endoconidia and chlamydospores. The cultures are dark green at the bottom and green at the upper side. Here too there is a tendency to sector forming.

The antagonist, *Penicillium roqueforti*, was cultivated in a Czapek medium containing 5 % saccharose, and of the culture filtrate 6 parallel series of dilutions with cherry juice were made, i.e. one series for each of the *Thielaviopsis* strains. The growth of the latter in the media consisting of cherry juice with various amounts of the culture filtrate of the antagonist was compared with its growth in control cultures on cherry juice without any addition of culture filtrate (Table 13).

TABLE 13

Growth of various strains of *Thielaviopsis basicola* in culture filtrates of *Penicillium roqueforti* that had been diluted with cherry juice

Strains of *Thielaviopsis basicola* isolated from:	Degree of dilution of the filtrate				
	1/32	1/64	1/128	1/256	1/512
Lathyrus odoratus	0	0	0	++	+++
Euphorbia pulcherrima	0	0	0	+++	+++
Nicotiana tabacum	0	0	0	+++	+++
Cypripedium spec.	0	0	+	++	+++
Primula obconica	0	0	0	++	+++
Nicotiana glutinosa	0	0	0	++	+++

Explanation of symbols, same as in Table 3.

The strain that had been obtained from the roots of *Cypripedium* showed some growth in the cultures which contained the filtrate in the dilution 1/128, but it was a very slight one. On the whole the various strains seem to be affected in the same way by the culture filtrate of *Penicillium roqueforti*.

So far the production of antibiotic substances by a number of soil fungi and the effect of these substances on the growth of *Thielaviopsis basicola* were studied in vitro. It appeared from the experiments that the growth-inhibiting activity of the filtrates obtained from the cultures of the various antagonists differed widely, and that the kind of culture medium and the nature and concentration of the carbon source exercised a considerable influence on the activity of the filtrates. The growth-inhibiting substance in the culture filtrates, with the exception of that in the filtrate of *Aspergillus fumigatus*, proved to be

resistent against steam sterilization for ten minutes at 103° C, and it was not affected by storage at room temperature for a period of 45 days.

Of the culture filtrates obtained from the antagonists that originally had been selected, those of *Gliocladium roseum* and of the sterile mycelium showed but a weak antibiotic activity, and these fungi were therefore discarded. The experiments with *Penicillium roqueforti*, on the other hand, looked promising. It appeared that the antibiotic activity of the culture filtrates of this fungus remained constant under constant environmental conditions. However, when this fungus was cultivated in media of different composition, the antibiotic activity proved to vary considerably. Because of this sensitivity to the composition of the medium *Penicillium roqueforti* was not used in the experiments on the behaviour of *Thielaviopsis* in soil which will be dealt with in the last chapter.

The antibiotic activity of the culture filtrates obtained from the other antagonists, viz. from *Aspergillus fumigatus*, *Penicillium spinulosum*, *Penicillium expansum* and *Penicillium spiculisporum*, showed less variability under differing circumstances. *Penicillium expansum* appeared to be the antagonist with the highest activity.

5. DEGREE OF SURVIVAL OF THELAVIOPSIS BASICOLA IN UNSTERILIZED SOIL

5.1. Relation between the natural microflora and the thielaviopsis population during a period of systematic infestation

It is well-known that many pathogenic fungi occurring in the soil, disappear in the long run or decrease in number (Katznelson, 1940). Physical factors and the activity of antagonistic micro-organisms are usually held responsible for this decrease. The fact that *Thielaviopsis basicola* is rather regularly found in soils (Yarwood, 1946; Stover, 1950b), proves that this root parasite does not disappear easily. It would therefore be interesting to know to what extent *Thielaviopsis* can maintain itself in a naturally infested soil as well as in a soil to which from time to time cultures of this fungus are added.

An investigation carried out to find an answer to this question would have to consider the following points:

(1) Does the number of *Thielaviopsis* colonies that can be isolated from a soil in which this fungus is naturally present, undergo a decrease in the course of time?

(2) What influence does a periodical addition of *Thielaviopsis* material exercise on the density of the population of the soil fungi that originally was present?

(3) What influence does such a periodical addition of *Thielaviopsis* exercise on the frequency of its antagonists?

(4) What is the influence which a periodical addition of *Thielaviopsis* to the soil exercises on the number of colonies of this fungus that can be isolated during this period of systematic infestation?

The two kinds of soil that were used in this series of experiments

were (a) a soil on which healthy plants of *Primula obconica* had been growing, and which consisted for 30 % of mud, for 20 % of leaf mould, for 20 % of clay, for 20 % of hog's dung and for 10 % of peat-dust; as no diseased plants had been observed on this soil, it will be designated here with the name "healthy soil"; and (b) "diseased soil", a soil on which severely infected plants of *Primula obconica* had been found, and which contained remains of *Primula* roots which on account of their infection by *Thielaviopsis basicola* had assumed a brown colour; this soil consisted for 30 % of mud, for 20 % of leaf mould and for 50 % of clay.

With each of the two kinds of soil 10 pots with a capacity of 1000 cc were filled to 1 cm below the brim, and in order to obtain a favourable environment for the development of fungi, the soil was moistened with sterilized water. In order to restrict the intervention of fungi from without, the pots were covered with plates of glass. Each of the two sets of pots was divided into two groups, indicated as "A" and "B"; those belonging to the groups "B" were subsequently infested with *Thielaviopsis*. All the pots were placed in a greenhouse where the temperature fluctuated between 20° C and 25° C. In order to keep the moisture content of the soil at a favourable level, the pots were embedded in peat-dust which was watered from time to time.

For the infestation of the pots belonging to the B groups with *Thielaviopsis*, use was made of suspensions of mycelium and spores. These suspensions were obtained from cultures in potato extract with 2 % glucose; when the latter were 14 days old, the mycelium was separated from the culture liquid and washed; after that 25 cc sterilized water was added. In this way a dense suspension of mycelium and spores was obtained.

Each of the pots belonging to the B groups received every other day 25 cc of this suspension, and each pot belonging to the A groups received at the same time an equal volume of sterilized water. In this way the soil in the pots belonging to the B group was regularly enriched with *Thielaviopsis*, whereas nothing was done which could change the composition of the microbiological population in the pots belonging to the A groups.

The composition of the microbiological population in the soil of the various pots was studied by means of soil samples. The latter were taken with intervals of two days from the 2nd March to the 22nd March, and in the pots belonging to the B groups this was done before a new *Thielaviopsis* suspension was added. Six days after the last administration of a *Thielaviopsis* suspension, i.e. on the 28th March, the soils in the pots were sampled once more. The samples were taken about 2–3 cm beneath the surface by means of a cork-borer, and weighed about 3 gm. Each sample was put in a sterilized test-tube.

The weight of the test tubes had previously been determined, and they were now weighed once more in order to obtain the exact weight of the amount of soil that had been placed in them. When

the weight had been determined, the soil was diluted 5000 times with sterilized water. This mixture was shaken for three quarter of an hour in a shaker in order to separate the soil particles from each other and to obtain a more even distribution of the mycelium and the spores.

An amount of 1 cc of each soil suspension was mixed with 15 cc molten cherry-agar at 40° C, and this mixture was poured out into a Petri dish, where it was allowed to solidify. At this dilution the number of fungus colonies that developed in each dish, fluctuated between 15 and 30.

In order to find out which of the fungi would be able to exercise an antagonistic effect on *Thielaviopsis*, each Petri dish was inoculated at 5 different places with this fungus, and as the observation of the antagonists is often obstructed by the fast growth of other fungi, and also because the counting of the number of colonies too may become difficult in this way, the dishes were kept at the relatively low temperature of 16° C.

The Petri dishes were regularly inspected in order to estimate the total number of fungus colonies, the number of colonies with an antagonistic effect on *Thielaviopsis* and the number of *Thielaviopsis* colonies derived from the soil (Table 15).

The figure that is given in this table for the number of fungus colonies, is the total number of colonies counted in a set of 5 Petri dishes, and as the colonies in the 5 dishes were obtained from 5 cc of a 1/5000 dilution of a certain weight of moist soil, the number per gm moist soil must be 1000 times as large. The figures in the table may therefore be multiplied by 1000.

It is rather remarkable that in the cultures obtained from the infested soils of group A, i.e. of the group to which no *Thielaviopsis* suspensions were administered, no *Thielaviopsis* colonies were found. As roots of *Primula obconica* infected with *Thielaviopsis* were present in large numbers in this soil, the most plausible explanation of the absence of *Thielaviopsis* colonies in the cultures seems to be that most of the chlamydospores and endoconidia occur in and on the diseased roots, and that but very few of them are set free in the soil. Thus, the first of the four questions that were formulated above, can not yet be answered. Although the parasite could not be isolated from the diseased soil of group A, it need not be doubted that it must have been present, not only because the primulas that in the past had been grown in this soil, had become diseased, but because those that were planted in it in the period following that of these experiments, also became infected (see next section). A decrease of the amount of the inoculum could therefore not be demonstrated.

In group A as well as in group B the number of fungus colonies obtained from the "diseased" soil was about 30 % higher than that obtained from the "healthy" soil, notwithstanding the latter seemed to be a more favourable substrate as it contained more organic matter. In group B the character of the fungus flora remained almost constant, in spite of the regular addition of the *Thielaviopsis* suspension. It

TABLE 15

Number of fungus colonies counted on five agar plates each inoculated with 1 cc of a suspension containing 1 gm soil in 5 l water

	Group A without addition of *Thielaviopsis basicola*				Group B with addition of *Thielaviopsis basicola* every other day									
	"healthy" soil		"diseased" soil		"healthy" soil					"diseased" soil				
	Number of fungus colonies	Number of antagonists	Number of fungus colonies	Number of antagonists	Number of fungus colonies other than *Thielaviopsis basicola*	Number of antagonists	Number of *Thielaviopsis basicola* colonies	Total number of fungus colonies	*Thielaviopsis basicola* colonies in % of total number	Number of fungus colonies other than *Thielaviopsis basicola*	Number of antagonists	Number of *Thielaviopsis basicola* colonies	Total number of fungus colonies	*Thielaviopsis basicola* colonies in % of total number
Date of sampling	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2/3	70	7	102	8	103	10	—	—	—	104	6	—	—	—
4/3	66	6	113	9	70	7	—	—	—	103	8	—	—	—
6/3	88	4	114	10	88	5	22	110	20.0	115	9	30	145	20.7
8/3	81	8	109	10	85	3	43	128	33.6	105	8	66	171	38.6
10/3	89	2	107	10	81	8	30	111	27.0	107	9	31	138	22.5
12/3	85	8	108	7	92	7	21	113	18.6	120	12	13	133	9.8
14/3	85	9	111	11	91	8	26	117	22.2	116	9	17	133	12.8
16/3	92	9	128	12	89	5	21	110	19.1	124	12	11	135	8.2
18/3	87	9	115	8	97	9	19	116	16.4	120	6	16	136	11.8
20/3	84	10	117	11	101	5	26	127	20.5	111	12	13	124	10.5
22/3	81	3	105	9	84	4	20	104	19.2	108	11	10	118	8.5
28/3	—	—	—	—	78	8	8	86	9.3	120	12	7	127	5.5
Total other than 28/3	908	75	1229	105	981	71	228	1036	—	1233	102	207	1233	—
Aver. other than 28/3	82.55	6.82	111.73	9.55	89.18	6.45	25.33	115.11	21.84	112.09	9.27	23.0	137.0	15.94

looked as if the microflora had reached a state of equilibrium which it tended to preserve against the attempts at interference. The answer to the second question is therefore that the density of the fungus population in the "diseased" as well as in the "healthy" soil was not noticeably affected by the regular introduction of new *Thielaviopsis* material.

More antagonistic colonies were isolated from the "diseased" soil than from the "healthy" soil of both groups. Therefore a stronger inhibition of *Thielaviopsis* was to be expected in the "diseased" soil. It is, on the other hand, rather unexpected that the number of colonies by which the antagonists are represented in the cultures isolated from the soil of the B group is of the same order of magnitude as the number of colonies of antagonists isolated from the soil of the A group. This means that the regular addition of *Thielaviopsis* inoculum does not lead to an increase in the density of the population formed by the antagonists. This, therefore, is the answer to the third question.

That the number of *Thielaviopsis* colonies in the cultures obtained from the soil of the B group gradually decreased, is doubtless a remarkable phenomenon; it shows that the density of the *Thielaviopsis* population which was reached after the first inoculations, could not be maintained. This decrease in density stands out more clearly when the figures in the columns 7, 9, 12 and 14 of table 15 referring to the isolations carried out in three consecutive periods are averaged, as in this way the influence of accidental differences is lessened (Table 16). This could, of course, not be done with the figures relating to the last isolations which were carried out on the 28th March, 6 days after the last infestation (22nd March).

TABLE 16

Number of *Thielaviopsis* colonies isolated from soils which from the 4th to the 22nd March were enriched every other day with suspensions of this fungus. The isolations were carried out every other day from the 6th to the 22nd March and once more on the 28th March

Isolations on	"Healthy soil"			"Diseased soil"		
	Total number of *Thielav.* colonies	Average number of *Thielav.* colonies	*Thielav.* colon. as % of total number of fungus colon.	Total number of *Thielav.* colonies	Average number of *Thielav.* colonies	*Thielav.* colon. as % of total number of fungus colon.
6.3; 8.3; 10.3	95	32	27.2	127	42	28.0
12.3; 14.3; 16.3	68	23	20.0	41	14	10.2
18.3; 20.3; 22.3	65	22	18.7	39	13	10.3
28.3	8	8	9.3	7	7	5.5

The number of *Thielaviopsis* colonies that in group B could be isolated from the diseased soil was at first somewhat higher than that which could be obtained from the healthy soil, but this lasted a short time only, and then the number that could be isolated from

the diseased soil sank to a value considerably below that obtained from the healthy one. It is noteworthy that in the cultures from the diseased soil the average number sank in the course of the first six days from 42 to 14, whereas in the healthy soil the average number sank in the same period from 32 to 23. When the number of *Thielaviopsis* colonies is expressed as a percentage of the total number of fungus colonies, it appears that this percentage is in the diseased soil about half that found in the healthy one. The higher density of the fungus population and especially the greater abundance of antagonists in the diseased soil may be responsible for this more rapid decrease of *Thielaviopsis*.

Although the number of *Thielaviopsis* colonies that could be isolated in the period 18/3–22/3 was slightly lower than that isolated in the period 12/3–16/3, the differences are so small that it seems justified to assume that a balance had been reached and that further addition of the *Thielaviopsis* suspension would have had no effect.

The fourth question therefore may be answered in this way that the number of *Thielaviopsis* colonies which can be isolated from "diseased" as well as from "healthy" soil when these soils are infested at regular intervals with this fungus, probably needs but a short time to reach a more or less constant value.

Six days after the administration of the *Thielaviopsis* suspension had been stopped, the number of colonies that could be re-isolated, showed a considerable decrease (Tables 15 and 16). The decrease was greater in the cultures obtained from the "healthy" soil (from 22 to 8) than in those obtained from the "diseased" one (from 13 to 7). The further history of the microflora of these soils was not studied.

When a micro-organism is regularly added to a soil, it is not unusual that this addition is followed by an increase in the development of its antagonists. In the investigations of which the results are recorded in this paper, it appeared that infestation of the soil with *Thielaviopsis basicola* does not cause a better development of its antagonists, but that, on the contrary, the development of *Thielaviopsis* is kept in check by the antagonists in their normal density and perhaps by the presence of other fungi which are part of the ordinary soil population.

5.2. THE PATHOGENICITY OF THIELAVIOPSIS BASICOLA IN THE SOILS OF WHICH THE MICROFLORA WAS STUDIED IN THE PRECEDING SECTION

The pots that had been used in the experiments described in the preceding section, were, after the experiments were finished, planted each with a young specimen of *Primula obconica* (height 3–4 cm) in order to test by the aid of the latter the pathogenicity of the *Thielaviopsis* population that at that moment was present. To this end the pots were kept for 104 days in a greenhouse at an average day temperature of 22° C. In each pot the pH of the soil was determined; the average values found for the 5 pots of which each set consisted, are recorded in the last column of Table 17. In the course of the

sojourn in the greenhouse the pH in the pots with the more strongly diseased plants showed a tendency to decrease, but the differences were so small that it is not probable that they would have influenced the growth of the plants.

In order to give an impression of the condition of the plants at the end of the experiment, in table 17 figures are collected with regard to the following features (1) total length of shoots, (2) total number of leaves ,(3) average length and (4) average width of leaves, (5) total number of inflorescences, (6) average length of inflorescences, (7) dry-weight of the shoots, and (8) dry-weight of the roots.

The condition of the plants that had been cultivated in the pots belonging to group A (Table 15), was as follows:

In the "healthy" soil the plants were healthy and well developed with large green leaves and a considerable number of inflorescences and flowers. The roots were healthy and well developed, white and without the symptoms of an infection by *Thielaviopsis*, abundantly ramified, long and distributed over a large area. The plants of this set were far better developed than those of the other ones.

In the "diseased" soil the plants were less well developed; the leaves were on the whole smaller, the inflorescences fewer in number, less well developed and provided with a smaller number of flowers. The roots too were less well developed and less well distributed in the soil; in many places they showed a brown discoloration and were rotten, although the top part was, as a rule, still white. In the infected parts of the roots a large amount of mycelium and of chlamydospores of *Thielaviopsis* was present.

The condition of the plants that had been grown in the pots belonging to group B (Table 15), was worse than that of the plants of group A that had been cultivated in the "diseased" soil.

The heaviest infection was found in the plants that had been grown in the "healthy" soil to which in this case at regular intervals a *Thielaviopsis* suspension had been administered. Nearly all the plants were on the verge of dying. The roots were thin and had hardly grown out, and they were almost entirely rotten and showed a brown to black discoloration.

In the "diseased" soil to which the *Thielaviopsis* suspension had regularly been administered, the plants had also badly developed, although on the whole somewhat better than those belonging to the previous set. The roots were weak and had hardly grown; for the greater part they were brown and rotten; they were moreover thin and brittle. The infected parts of the roots contained large amounts of chlamydospores. The fungus could be isolated from them. The infection was doubtless not so severe as in the preceding set.

It appears from Table 17 that the plants in the not-infested "healthy" soil showed by far the best development, and as these plants did not show a single symptom of the disease, they can be regarded as a control set.

The most severe infection was found in the two sets of plants belonging to the group B, i.e. to the plants that grew in a soil that

TABLE 17

Condition of *Primula obconica* plants after 3 months growth in "healthy" and in "deseased" soil that had been left as it was (group A) or to which several times a *Thielaviopsis* suspension had been added (group B)

	Total length of shoots in cm	Total number of leaves	Average length of leaves in cm	Average width of leaves in cm	Total number of inflorescences	Average length of inflorescences in cm	Dry-weight of shoots in gm	Dry-weight of of roots in gm	pH of the soil
	1	2	3	4	5	6	7	8	9
Group A:									
"Healthy" soil	1607.0	79	15.14	8.12	27	15.23	23.48	2.42	6.9
"Diseased" soil	651.0	51	10.32	6.14	9	13.88	9.30	1.70	6.7
Group B:									
"Healthy" soil repeatedly infested with *Thielaviopsis*	250.0	22	9.02	5.05	5	10.30	3.27	0.42	6.4
"Diseased" soil repeatedly infested with *Thielaviopsis*	311.0	27	9.90	5.40	3	14.63	4.03	0.70	6.5

had been infested at regular intervals; the plants that grew in the "healthy" soil were most strongly diseased.

To some extent the results that were obtained by the investigations with regard to the development of *Thielaviopsis* in non-sterilized soil, and that are summarized in the Tables 15 and 16, are reflected in those of the two last-mentioned sets of experiments with *Primula obconica*, as in these earlier experiments the *Thielaviopsis* population underwent in the "diseased" soil a stronger decrease in density, and reached a lower final level. Although the fungus could not be isolated from the "diseased" soil to which no suspension of *Thielaviopsis* had been administered, the plants that were grown in this soil nevertheless became severely infected, and although a direct proof of the presence of the fungus could in this case not be given, its presence could convincingly be demonstrated by the aid of a susceptible host.

6. INOCULATION EXPERIMENTS WITH NICOTIANA GLUTINOSA

6.1. Influence of physiogenic factors and of the presence of thielaviopsis in the soil on the development of nicotiana glutinosa

It is well-known that the cultivation of *Nicotiana glutinosa* in a glasshouse may offer difficulties. A yellowing of the leaves and even a premature dying may occur, and sometimes the plants are weakened by an attack of *Thielaviopsis*. Unfavourable physiogenic factors and the presence of the fungus in the soil may cooperate to cause a syndrome of symptoms which is difficult to disentangle. An accurate assessment of the pathogenicity of *Thielaviopsis* is possible only when

the harm caused by unfavourable external conditions is eliminated. To this end it seemed desirable to determine first of all under what conditions a healthy development of the plants may be obtained.

As temperature, light intensity and air humidity seemed to be the factors that deserved special attention, the experiments were confined to them. The influence of each of them was tested at two levels, viz.

Temperature:	25° C (1)	or	19° C (2)
Light intensity:	sunlight (3)	or	shade (4)
Air humidity:	r.h. 95–100 (5)	or	r.h. 25–60 (6)

Combination of these conditions is possible in 8 ways, viz.

1. 3. 5.	1. 3. 6.	1. 4. 5.	1. 4. 6.
2. 3. 5.	2. 3. 6.	2. 4. 5.	2. 4. 6.

and all these combinations were tested.

The experiments were carried out in a period of 28 days, viz. from April 15th to May 12th. The average day length during this period was 14.40 hours. The plants were grown in sterilized garden soil of which the pH varied between 5.0 and 5.4, and for each of the 8 combinations of conditions two sets of 16 plants each were used, one of the sets of 16 being infested with *Thielaviopsis*, and the other set serving as control. The infestation was effected by means of spore suspensions, which were mixed as thoroughly as possible with the soil. The quantity of inoculum was approximately 3000 to 3500 infection units per cc soil; these units were partly conidia and partly chlamydospores. The choice of this quantity was based on the findings of Levykh (1938). The experiments were carried out with young, healthy plants, 5–7 cm in height, and for the various sets equivalent ones were chosen. The two sets that were used for each combination of conditions were placed together in a glass chamber.

In order to obtain the relative humidity of 95–100 (5) in sunlight (3), the plants were artificially misted. For the plants in the shade the same degree of humidity was obtained by clothing the glass chambers on the inside with filter-paper and keeping the latter moist, and by covering the chambers with a glass plate placed on moist filter-paper. The water content of the soil was always kept more or less at the same level. Shading was effected by means of filter-paper by which the sunlight was shut out as much as possible, without, however, causing a noticeable rise of the relative humidity. As the sky was hardly ever overcast, the circumstances were favourable for the study of the effect of sunlight.

After about 20 days the condition of the infected plants that were exposed to direct sunlight, was getting worse and worse, and after 28 days they were on the verge of dying. In order to obtain properly comparable results, the experiment should be ended before any plant had succumbed.

At the end of the experiment all the plants were appraised according to a "condition index". In the latter the figure 1 was given to the

weakest plants found in the 16 sets, and the figure 10 to the healthiest and best developed ones; the rest of the plants were awarded figures between these extremes. This mode of assessment made it possible to obtain a rather reliable picture of the differences between the various sets.

The "condition figures" assigned to each of the 16 plants of a set have been added, and these sums are for each of the 16 sets of plants set down in the graphs reproduced in Fig. 1.

The experiment shows that the condition by which the development of *Nicotiana glutinosa* is most unfavourably influenced, is the exposition

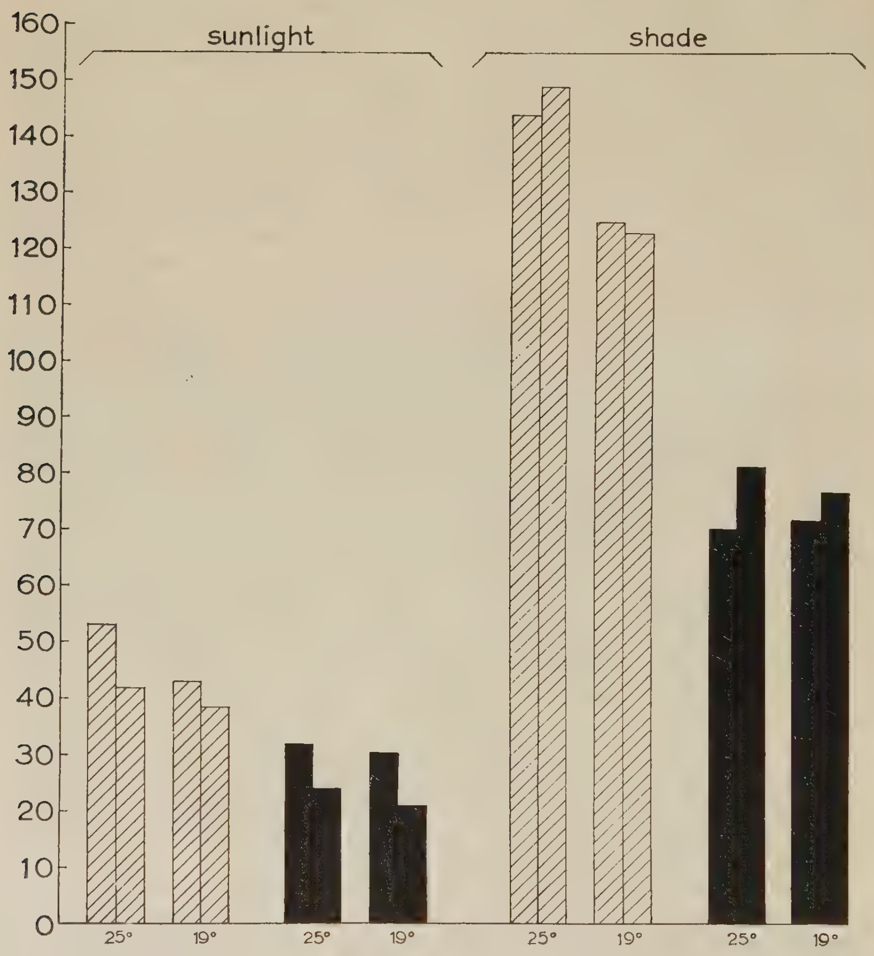

Fig. 1. Total "condition index" of *Nicotiana glutinosa* plants after 28 days of growth in infested (black) and uninfested soil (shaded), and under influence of various external factors.

The left column of each pair refers to a high humidity, the right one to a low humidity.

to full sunlight. The latter caused a poor growth; the plants were dwarfed, and flowering started at an early stage. Moreover, the leaves showed a yellow discoloration, particularly around the veins; they had therefore a mottled look. The plants nevertheless succeeded in completing their life cycle.

With regard to the influence of the temperature it should be noted that at the beginning the plants grew noticeably better at 25° C than at 19° C. However, towards the end of the experiment the difference became less pronounced. The best developed plants were those that had been grown at 25° C in the shade; the weakest ones were those that at 19° C had been exposed to sunlight and to a low relative humidity. Under the conditions of this experiment temperature may be regarded as the condition that is second in importance.

Low air humidity affected the plants only when they were grown in sunlight, and then at the higher as well as at the lower temperature. The plants that were grown in the shade, proved to be indifferent to the humidity of the air. In this experiment the last-mentioned condition seemed to be of least importance.

In the infested soil all plants proved to be attacked by the parasite, although the degree of infection varied with the combination of the physiogenic conditions. The plants grown in direct sunlight showed a syndrome that was partly caused by the physiogenic conditions and partly by the infection, whereas the plants that were grown in the shade, were damaged only by the parasite. In the infected plants the leaves, especially the lower ones, showed either a more or less even yellowing or a withering, depending upon the severity of the attack, and, in contrast to the leaves that showed a yellow discoloration because of their exposition to direct sunlight, the yellow leaves of these plants withered shortly afterwards.

The condition index of the infected plants that had been kept in the shade, was much higher than that of the corresponding plants in direct sunlight; that of the worst set in the shade was still higher than that of the best set of the not-infected plants that had been exposed to direct sunlight. However, when the condition index of the shaded plants is compared with that of the sunlight-plants, it appears that the decrease caused by the infection is in the first-mentioned group of plants greater than it is in the latter.

In the shaded plants the difference in development between the infected and the not-infected ones appeared to be larger in the sets that had been grown at a temperature of 25° C than it was in the corresponding sets that had been grown at 19° C. It appears that not only the development of *Nicotiana glutinosa* is favoured by the higher temperature, but also that of the parasite. This is in agreement with earlier findings according to which the optimum temperature for the growth of *Thielaviopsis* comes nearer to 25° C than to 19° C.

The bad condition of the plants that had developed in direct sunlight has previously led to the assumption that sunlight favours the development of black root-rot in *Nicotiana glutinosa*. This experiment, however, shows that shaded plants are actually more severely

damaged by the parasite than the plants grown in sunlight. For an accurate assessment of the pathogenicity of *Thielaviopsis* it is therefore inadvisable to grow *Nicotiana glutinosa* in direct sunlight, since the influence of the parasite is in that case obscured by that of the exposition to direct sunlight. However, under all the conditions tested in this experiment *Nicotiana glutinosa* proved to be very susceptible to the attacks of the parasite, a fact that was already mentioned by Johnson in 1916.

The different conditions under which *Nicotiana glutinosa* was grown, appeared to have no influence on the development of the root system. Differences in the development of the latter were always due to the presence or absence of the parasite. In the not-infected plants the roots always were healthy, white and more or less evenly distributed through the soil. The roots of the infected plants, on the other hand, were poorly developed and spread but imperfectly in the soil; they were mostly brown, and several of them were dead and rotten. Occasionally the roots appeared to be somewhat better developed, but even then their development did not correspond to that of the aerial parts. In the diseased roots the chlamydospores of *Thielaviopsis* were present in large amounts; from these roots the fungus could be isolated.

When the infected plants that had been grown in the shade, either at a temperature of 19° C or at 25° C, were exposed to sunlight and to a somewhat drier air, they wilted within ten minutes, whereas the not-infected plants remained fresh, even when the exposure to sunlight and drier air was extended to one hour. This points to a retardation either of the water uptake or of the water transport in the infected plants, a retardation for which *Thielaviopsis* is very likely responsible. Toxic substances secreted by the parasite might be directly involved.

The response of *Nicotiana glutinosa* to the two temperatures tested in this experiment is not the same as that found by other investigators in *Nicotiana tabacum*. In the latter the temperature exercised a stronger influence on the degree of infection. However, it is not excluded that the influence of the temperature on the infection of *Nicotiana glutinosa* would have been more marked when the amount of *Thielaviopsis* inoculum had been smaller.

The pH of the soil varied, as stated above, between 5.10 and 5.40, but as this must be regarded as a rather narrow range, and as the variation, moreover, was not related to differences in the external conditions, it may be assumed that it did not affect the development of the host plants nor that of the parasite. For *Nicotiana tabacum*, however, the significance of the pH of the soil for the development of black root-rot has been recognized more than once and by various investigators.

In all the combinations of light, temperature and air humidity that were tested in this experiment, the disease developed at a pH lower than 5.5, whereas according to Doran (1929) and others in *Nicotiana tabacum* black root-rot is never met with at a pH lower

than 5.6. This difference may, however, partly or entirely be due to the fact that in the experiment with *Nicotiana glutinosa* a much larger amount of *Thielaviopsis* inoculum was used than in Doran's experiments with *Nicotiana tabacum*. In this connection it should be remembered that according to Johnson and Hartman (1919) the resistance of acid soils against the infection with *Thielaviopsis* may be overcome completely, at least if a susceptible variety of *Nicotiana tabacum* is used, by increasing the amount of inoculum.

In another section of the greenhouse in which the experiment with *Nicotiana glutinosa* was carried out, more plants of this species and also plants of *Nicotiana tabacum* were grown for other purposes. These plants grew in the same soil as the plants that were used for the experiment, i.e. at a pH lower than 5.6, but this soil had not been sterilized and was naturally infested with *Thielaviopsis*. The temperature in this section was approximately 25° C, and the *Nicotiana glutinosa* plants were protected from the direct sunlight, those of *Nicotiana tabacum* not. In the latter no signs of black root-rot were ever observed, and this is in good agreement with the experiences of other investigators, but in nearly all the plants of *Nicotiana glutinosa* the infection was clearly discernible, especially during the later stages of their development. This observation therefore confirms the view that in *Nicotiana glutinosa* a higher temperature and a pH lower than 5.6 do not control the black root-rot to the same extent as they do in *Nicotiana tabacum*.

6.2. Influence of some antagonists upon the pathogenicity of thielaviopsis basicola

The experiment described in the preceding section showed that for an assessment of the pathogenicity of *Thielaviopsis basicola* by means of *Nicotiana glutinosa* the latter should not be grown in direct sunlight, as in that case the syndrome caused by this mode of cultivation may obscure the symptoms of the disease. The experiment also gave indications with regard to the influence of temperature and air humidity, and on account of these indications the following combination of conditions was chosen to carry out some investigations on the influence exercised by antagonists on the pathogenicity of *Thielaviopsis*: a temperature of 25° C, shade and a high humidity. The fungi whose antagonistic activity was tested, were *Penicillium spiculisporum*, *Penicillium expansum*, *Aspergillus fumigatus* and *Penicillium spinulosum*.

For this investigation the following sets of plants were used:

A. Plants grown *in sterilized soil* with a pH of 5.52–5.98
- *a.* Soil not infested
- *b.* ,, infested with *Thielaviopsis*
- *c.* ,, ,, ,, *Thielaviopsis* + *Penicillium spiculisporum*
- *d.* ,, ,, ,, *Penicillium spiculisporum*
- *e.* ,, ,, ,, *Thielaviopsis* + *Penicillium expansum*

f. ,, ,, ,, *Penicillium expansum*
g. ,, ,, ,, *Thielaviopsis* + *Aspergillus fumigatus*
h. Soil infested with *Aspergillus fumigatus*
i. ,, ,, ,, *Thielaviopsis* + *Penicillium spinulosum*
j. ,, ,, ,, *Penicillium spinulosum*

B. Plants grown *in unsterilized soil*
k. Soil infested with *Thielaviopsis* (pH 6.65)
l. ,, not infested (pH 6.16).

The sets ***a***, ***b*** and ***l*** served as controls.

The number of plants per set was 20; it were young, vigorously growing plants with a height of 6–8 cm, and they were distributed in such a way over the sets that the latter were all of similar composition.

The infection was carried out in the same way as in the experiment described in the preceding section, i.e. by means of spore suspensions. Where *Thielaviopsis* was used in conjunction with an antagonist, the two spore suspensions were thoroughly mixed before they were added to the soil. The amount of *Thielaviopsis* inoculum was the same as in the previous experiment, viz. 3000–3500 infection units per cc soil. Of the antagonist 3000–5000 spores per cc soil were used.

The experiments were carried out in the period May 28th to June 17th, and the average day length in this period was 16 hours 29 minutes.

In contrast with the 28 days of the previous experiment, this set of experiments lasted but 20 days. At the end of this period the plants were already further developed then in the corresponding sets of the previous experiment and the symptoms of the disease were much more pronounced. It seems plausible to assume that the development required one third less time. The cause of this more rapid development of the host as well as of the symptoms of the disease can not be found in differences in temperature, light intensity, air humidity, soil features or the amount of inoculum, as all these conditions were the same. The plants with which this set of experiments were started, were somewhat larger, but this difference can hardly have been of importance. The pH of the soil was approximately 0.5 higher, and this may have favoured the development of the parasite, but hardly that of the host. The average day length was 1 hour 49 minutes longer, and it is not impossible that this factor may have been responsible for a more rapid growth. Stover (1950b) is of opinion that the average day length is of importance for the pathogenicity of *Thielaviopsis* in tobacco plants. According to him a minimum day length of 12 hours is required.

After approximately 10 days the plants in the sterilized soil that had been infested with *Thielaviopsis* alone, appeared to be heavily attacked. They showed the symptoms of the disease, and lagged far behind the control plants. At this stage there was as yet hardly any

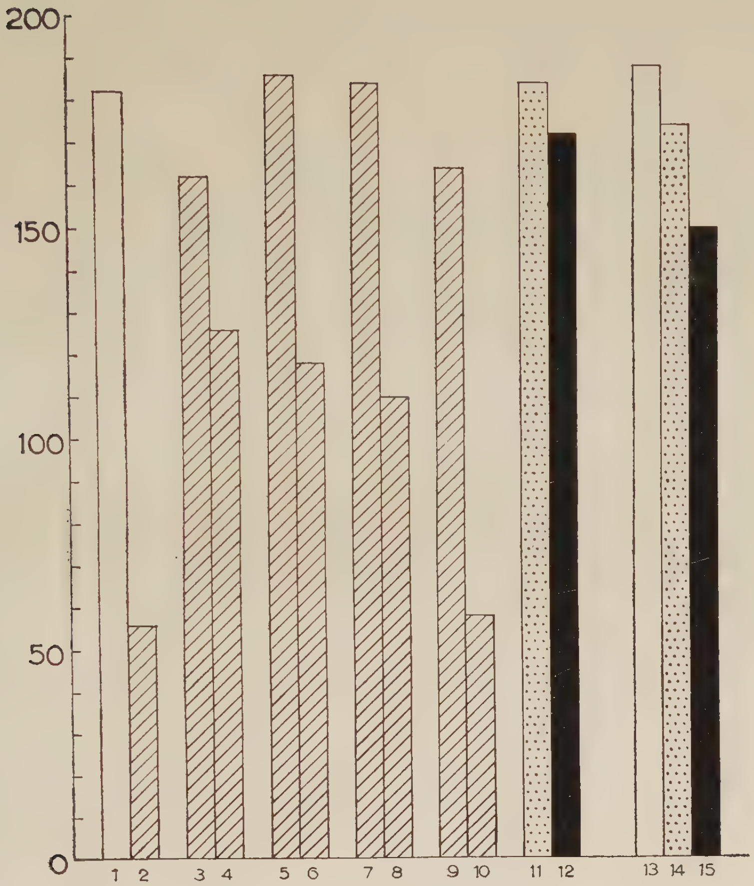

Fig. 2. "Condition index" of *Nicotiana glutinosa* plants after 20 days growth (1–12) and after 35 days growth (13–15) in sterilized soil (1–10 and 13) and in unsterilized soil (11, 12, 14 and 15).

1 = not infested
2 = infested with *Thielaviopsis basicola*
3 = ,, ,, *Penicillium expansum*
4 = ,, ,, *Thielaviopsis* + *Penicillium expansum*
5 = ,, ,, *Penicillium spiculisporum*
6 = ,, ,, *Thielaviopsis* + *Penicillium spiculisporum*
7 = ,, ,, *Penicillium spinulosum*
8 = ,, ,, *Thielaviopsis* + *Penicillium spinulosum*
9 = ,, ,, *Aspergillus fumigatus*
10 = ,, ,, *Thielaviopsis* + *Aspergillus fumigatus*
11 = unsterilized soil uninfested
12 = unsterilized soil infested with *Thielaviopsis*
13 14 and 15 = same as 1, 11 and 12 but assessed 15 days later.

difference between the other sets of plants, with the exception of that in the soil which had been inoculated with the mixture of *Thielaviopsis* and *Aspergillus fumigatus*. It looked therefore as if the antagonists had been able to keep the infection with *Thielaviopsis* in check. However, in the course of the next 10 days the plants of the sets growing in soil that had been inoculated with a mixture of *Thielaviopsis* and one of the three *Penicillium* species became also distinctly infected by the parasite, although not to the same degree as the plants in the sterilized soil that had been infested with *Thielaviopsis* alone and in that which had been inoculated with the mixture of the latter and *Aspergillus fumigatus*; these plants were on the verge of dying.

On the 20th day the experiment was ended, and the plants of each set were assessed according to a "condition-index" similar to that used in the previous experiment (Fig. 2). With the two sets growing in the unsterilized soil (11 and 12), in which the plants showed hardly any symptoms of the disease, and with the set in the sterilized soil that had not been infested with *Thielaviopsis* (1), the experiment was continued for another fortnight.

Penicillium expansum, *Penicillium spiculisporum* and *Penicillium spinulosum* appeared to exert an inhibiting effect on the pathogenicity of *Thielaviopsis*, but *Aspergillus fumigatus* failed to do so. This fungus and *Penicillium expansum* adversely affected the development of *Nicotiana glutinosa*, while *Penicillium spiculisporum* and *Penicillium spinulosum* hardly exerted any influence.

In the unsterilized soil the natural microflora appeared to have exerted a strong inhibiting effect on the pathogenicity of *Thielaviopsis*, despite the fact that the soil was severely infected with the latter and in spite of the addition of a suspension of its spores.

At the end of the experiment the control plants in the unsterilized soil (11) looked, despite the natural infestation of the soil with *Thielaviopsis*, better than the control plants in the sterilized soil that had not been inoculated with this fungus (1). In unsterilized soil that had been inoculated with *Thielaviopsis* (12), *Nicotiana glutinosa* grew better than it did in the sterilized soil that had been inoculated with a non-parasitic soil fungus such as *Penicillium expansum* (3) or *Aspergillus fumigatus* (9).

The final condition-index of the plants belonging to the sets with which the experiment was continued for a fortnight (1, 11 and 12) is given also in Fig. 2 (13, 14 and 15).

The plants in the sterilized soil (13) showed the highest index, directly followed by those in the unsterilized soil. In the set growing in the unsterilized soil to which *Thielaviopsis* had been added, the index appeared to have decreased in this fortnight (12 and 15), but it was still higher than that which had been found in the sets grown in sterilized soil to which the mixture of the spores of *Thielaviopsis* and of one of the antagonists had been administered (4, 6, 8 and 10). Even *Penicillium expansum* (4) appeared to have a less strongly inhibiting effect than the natural microflora of this garden soil.

If the assessment in all sets had been postponed till the end of the

extra fortnight, the difference between the plants in the sterilized soil to which *Thielaviopsis* had been added and those in the unsterilized one which had been inoculated with this fungus would have been much greater, since the development of the plants in the former lagged, throughout the whole period, behind that of the plants in the latter.

In the various sets that were grown in sterilized soil the condition of the roots proved to correspond more or less with that of the aerial parts. In the soil that had not been infested with *Thielaviopsis* (1), the roots were healthy and well developed. In the soil that had been inoculated with *Thielaviopsis* alone (2), the roots were severely infected; they ranged from poorly developed to completely shrivelled, and almost all were brown and had rotten. More or less the same condition was found in the roots that had developed in the soil which had been inoculated with the mixture of *Thielaviopsis* and *Aspergillus fumigatus* (10). In the soil that had been infested with the mixture of *Thielaviopsis* and *Penicillium spiculisporum* (6) and in that where the infestation had been performed with the mixture of *Thielaviopsis* and *Penicillium expansum* (4), the roots proved to be affected by the parasite, and an intensive brown discoloration and rotting had taken place, but their condition was nevertheless much better, ranging from adequate to good, and they were less severely diseased than those of the plants in the soil inoculated with *Thielaviopsis* without an accompanying antagonist (2). In the soil that had been inoculated with the mixture of *Thielaviopsis* and *Penicillium spinulosum* (8) the roots were considerably more infected and worse developed than in the sets 4 and 6, in which the two other *Penicillium* species had been used as antagonists, but they were better developed than in the sets 2 and 10, i.e. in the soil that had been inoculated with *Thielaviopsis* without the addition of an antagonist and in that in which *Aspergillus fumigatus* had to play the part of the antagonist. In the other sets the roots were healthy and well developed, although there were small differences, those in the soil that had been inoculated with *Aspergillus fumigatus* (9) and those in that with *Penicillium expansum* (3) being somewhat less well developed.

In the unsterilized soil the roots of the control plants (14) showed an extensive brown discoloration accompanied by rotting, but they were nevertheless well developed, and at the base of the stem many new and healthy roots were growing out. In the set that developed in the soil that had been inoculated with *Thielaviopsis* (12), the roots were far more strongly infected and more poorly developed.

In the sterilized soil the pH varied between 5.52 and 5.98, and, as in the experiment described in the preceding section, there appeared to be no correlation with the condition of the *Nicotiana glutinosa* plants. This pH was higher than in the previous experiment, where it varied between 5.10 and 5.40, and its range partly overlapped the "critical region" (pH 5.6–5.9) in which, according to Anderson, Osmun and Doran (1926) and Doran (1929), black root-rot is almost certain to cause trouble. In the unsterilized soil the pH was even higher, viz. 6.15 and 6.65, and here the plants were nevertheless not

so strongly affected as in the sterilized soil with the lower pH. It appears therefore that the natural microflora of the soil exerted a more strongly inhibiting effect upon the pathogenicity of *Thielaviopsis* than the antagonists individually did in a soil with a pH that was less favourable to the development of the parasite.

The experiments with four different antagonists described in this section revealed that *Penicillium expansum* exercised the strongest antagonistic effect on *Thielaviopsis*, and that it is followed by *Penicillium spiculisporum* and *Penicillium spinulosum*. This result is in agreement with that obtained by the experiments in vitro (Table 4). The natural microflora occurring in a garden soil may exercise an even more important influence on the pathogenicity of *Thielaviopsis basicola*. As this root parasite is generally present in garden soil, it obviously does not disappear from the latter under the influence of other micro-organisms; however, it may be kept in check by the latter.

SUMMARY

That the damage which *Thielaviopsis basicola* (Berk. et Br.) Ferraris may cause to a definite host plant, is not always equally severe, has been known already for a long time. This variability has usually been ascribed to physical factors operating in the soil, but another circumstance might also be of importance, viz. the presence of micro-organisms acting as antagonists. It is rather striking that the influence which such organisms might exercise on *Thielaviopsis basicola*, has received so far little or no attention, and for this reason a study of this problem in vitro as well as in the soil seemed appropriate.

Thielaviopsis basicola was isolated from the roots of *Primula obconica* as well as from those of *Nicotiana glutinosa*, and by using the isolation method of Yarwood (1946) it was found that at Baarn (Netherlands) this root parasite is very common in garden soil.

In order to obtain antagonists, samples of various kinds of soil were plated out, and the fungi that developed on the plates, were isolated and tested as to their power to inhibit the growth of *Thielaviopsis*. On cherry agar about 38 of them showed an antagonistic effect. In further experiments the antagonistic activity of these fungi was estimated by means of filtrates obtained from cultures in cherry juice. In this way about 20 % of the 38 fungi were found to cause in *Thielaviopsis* a notable growth inhibition. The strongest antagonistic activity was found in the culture filtrates of *Aspergillus fumigatus* Fres., *Penicillium expansum* (Link) Thom, *Penicillium spinulosum* (Link) Thom, *Penicillium spiculisporum* Lehman and *Penicillium roqueforti* Thom. The two last-mentioned species were so far unknown as producers of antibiotic substances.

The composition of the culture medium in which the fungi were grown, appeared to exercise a marked influence on the antibiotic activity of the culture filtrates as observed in cultures of *Thielaviopsis*. From a Czapek–Dox medium, in most instances, a more active filtrate was obtained than from a cherry-juice medium or from a culture in potato extract. In the Czapek medium, moreover, the carbon source proved to be of importance; in the case of *Penicillium roqueforti* saccharose gave the most active filtrate, whereas with *Penicillium spiculisporum* glucose and maltose proved to be more suitable. It was found, moreover, that in the case of *Penicillium roqueforti* the activity of the filtrates increased when the concentration of the saccharose in the Czapek medium was raised from 1 % to 5 %. The antibiotic activity of the filtrate of this fungus appeared to decrease when corn steep liquor was present.

The growth-inhibiting substances in the culture filtrates, with the exception

of those present in the filtrate of *Aspergillus fumigatus*, proved to be able to withstand a temperature of 103° C for 10 minutes; at room temperature they retained their activity for at least 45 days.

Penicllium roqueforti proved to produce its growth-inhibiting substance(s) mainly in the period of active growth, i.e. during the first 15 days. When the fungus was kept for more than 30 days on the same medium, the antibiotic activity of the filtrate decreased.

Different strains of *Penicillium roqueforti* were found to differ as to the inhibiting effect of their culture filtrates, whereas different strains of *Thielaviopsis basicola* reacted more or less in the same way on the culture filtrate of this antagonist.

The relation between the natural microflora of different soils and the development of *Thielaviopsis* in the latter were also studied. To this end the soils were periodically inoculated with a suspension obtained from a *Thielaviopsis* culture. Two different soils were tested, viz. a "diseased" soil, i.e. a soil in which diseased plants of *Primula obconica* had grown, and a "healthy" soil, i.e. a soil in which the Primulas had remained healthy. In the "diseased" soil, at the beginning of the experiment, the presence of *Thielaviopsis* could not be demonstrated by means of the plate method; that it nevertheless was present, follows from the fact that Primulas which were subsequently planted in this soil also became infected. Suspensions containing the mycelium and the spores of *Thielaviopsis* were added every other day, from the 4th March to the 22nd March, and every time before a new dose was given, a soil sample was taken in which the composition of the microflora was quantitatively determined. It appeared that the frequency of the soil fungi was not influenced by the periodic inoculations with *Thielaviopsis*. Contrary to the expectation, the frequency of the antagonists too remained the same.

During the first 6 days of the experiment the number of *Thielaviopsis* colonies remained high, but then a sharp decrease was noted. It was largest in the soil in which the microflora was best developed, i.e. in the "diseased" soil. During the later part of the experiment the lower level was maintained. Shortly after the additions of the *Thielaviopsis* suspension were stopped, the number of *Thielaviopsis* colonies in the soil samples decreased with 50 to 60 %.

When the experiment was ended, the soil was planted with *Primula obconica*, and then it appeared that the infection of the latter was heaviest in the "healthy" soil that had periodically been inoculated with *Thielaviopsis*. This is in agreement with the finding that the number of *Thielaviopsis* colonies that could be isolated from the "diseased" soil underwent a stronger decrease than the number that could be isolated from the "healthy" soil. The experiment with the Primulas is also of interest because it shows that the presence of a parasite can sometimes be demonstrated by means of a susceptible host where attempts to isolate it by means of the plate method remain unsuccessful.

In the experiments on the influence exercised by various antagonists on the pathogenicity of *Thielaviopsis*, *Nicotiana glutinosa* was used as a test plant. It is very susceptible to infection by *Thielaviopsis*.

First the influence exercised by various external conditions on the growth of *Nicotiana glutinosa* and on its infection by *Thielaviopsis* was investigated in order to find a suitable combination of conditions for the cultivation of this plant, i.e. a combination that does not cause a syndrome by which the symptoms of the disease might be obscured. It appeared that in direct sunlight the plants grew slowly and remained stunted; the leaves showed a yellow discoloration, and flowering started at an early stage. These symptoms may easily be mistaken for those caused by an infection with *Thielaviopsis*. A temperature of 25° C proved to be slightly better than a temperature of 19° C, but the humidity of the atmosphere had hardly any effect.

The degree to which the roots were infected, showed little or no correlation with the external conditions, but this did not apply to the plant as a whole, for the infected plants that had developed in direct sunlight were at the end of the experiment at the verge of death, whereas the infected plants that were grown in the shade, though they had lost a good deal of their vigour, were still in a fairly good condition. The "condition-index" calculated for the two groups shows, on the other hand, that the decrease in vigour shown by the shaded plants was proportionally larger than that of the sunlight plants (Fig. 1).

The amount of inoculum that is required for a severe infection of *Nicotiana glutinosa*, proved to be 3000–3500 infection units (chlamydospores and conidia) per cc soil. A dose of this strength was used for the assessment of the pathogenicity of this root parasite in sterilized soils to which an antagonist had been added, and also in an unsterilized soil, i.e. in a soil in which the natural microflora still was present. The conditions under which this experiment was carried out, were shade, a temperature of 25° C and a high relative humidity, because this are the circumstances which proved to be not only favourable for the development of the plants, but which also allowed an accurate assessment of the severity of the disease symptoms caused by *Thielaviopsis basicola* in the presence or absence of antagonists.

In sterilized soil the infection of *Nicotiana glutinosa* by *Thielaviopsis* proved to be inhibited to some extent when the soil was at the same time infested with *Penicillium expansum*, *Penicillium spiculisporum* or *Penicillium spinulosum*. *Aspergillus fumigatus* had no effect. The experiment with unsterilized soil showed that the inhibition exercised by the microflora which is normally present in the soil, is far more effective than that which could be obtained in a sterilized soil by inoculation with one of the above-mentioned antagonists (Fig. 2). Of all the antagonists tested, the three *Penicillia* proved to be most effective against *Thielaviopsis basicola* in the soil as well as in vitro.

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